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INDEX OF AUTHORS

PAGE
ALEEM, A. A. The recent migration of certain Indo-Pacific algae from the Red Sea into the Mediterranean 88
ASHBY, ERIC. Studies in the morphogenesis of leaves. I. An essay on leaf shape . 153
—— Studies in the morphogenesis of leaves. II. The area, cell size and cell number of leaves of *Ipomaea* in relation to their position on the shoot 177
AUDUS, L. J. Studies on the phytostatic action of 2:4-dichlorophenoxyacetic acid and coumarin. The reversibility of root-growth inhibitions 196
—— Hormones and Horticulture. The use of special chemicals in the control of plant growth (Review) 298
BAKER, H. G. The ecotypes of *Melandrium dioicum* (L. emend.) Coss. & Germ. . 131
BOND, T. E. T. The Nature and Prevention of the Cereal Rusts as exemplified in the Leaf Rust of Wheat (Review) 147
—— Plant Viruses (Review) 300
BRIGGS, G. E. and ROBERTSON, R. N. Diffusion and absorption in disks of plant tissue 265
BURTON, W. G. and SPRAGG, W. T. An apparatus for the extraction of gas from small amounts of plant sap or tissue 17
CANTER, HILDA M. and LUND, J. W. G. Studies on plankton parasites. I. Fluctuations in the numbers of *Asterionella formosa* Hass. in relation to fungal epidemics . . 238
CONWAY, VERONA M. Von Post's work on climatic rhythms 220
CORNER, E. J. H. Studies in the basidium. I. The ampoule effect, with a note on nomenclature 22
GILMOUR, J. L. The Royal Botanical Expedition to New Spain (Review) 296
GODWIN, H. The Living Plant (Review) 297
HOVASSE, R. (*see* Pringsheim, E. G.)
HUSEIN SAÏD. The effect of stem-ringing on the daily march of stomatal rhythm in *Kalanchoë* leaves 288
—— and TOLBA, M. K. The effect on stomatal behaviour of detaching leaves of *Kalanchoë* and *Mesembryanthemum* at different times of the day 28
LUND, J. W. G. (*see* Canter, Hilda M.)
METCALFE, G. The Chemical Activities of Bacteria (Review) 29
MITCHELL, G. F. Late-glacial deposits in Berwickshire 26

PAGE

PRINGSHEIM, E. G. The loss of chromatophores in *Euglena gracilis*. With a cytological contribution by Hovasse, R. 51

RICHES, J. P. R. An introduction to polarographic methods and their application to the analysis of plant material 1

ROBERTSON, R. N. (*see* Briggs, G. E.)

SAÏD, HUSEIN (*see* Husein Saïd)

SPORNE, KENNETH R. A note on a rapid clearing technique of wide application . 290

SPRAGG, W. T. (*see* Burton, W. G.)

TANSLEY, A. G. The nature and range of variation in the floral symmetry of *Potentilla erecta* (L.) Hampe 95

THODAY, J. M. A note on cell division in the desmid *Cosmarium meneghinii* . . 292

—— Heredity and Variation in Micro-organisms (Review) 295

TOLBA, M. K. (*see* Husein Saïd)

TURRILL, W. B. The genus *Crepis*, Parts I and II (Review) 146

VALENTINE, D. H. Studies in the British Primulas. II. Ecology and taxonomy of Primrose and Oxlip (*Primula vulgaris* Huds. and *P. elatior* Schreb.) . . . 111

WALTERS, S. M. Den Skogliga Rasforskningen och Praktiken (Review) . . . 148

WHITEHOUSE, H. L. K. The Genetics of Garden Plants (Review) 299

YEMM, E. W. Plant Physiology (Review) 298

INDEX OF SUBJECTS

PAGE

Absorption, Diffusion and, in disks of plant tissue—G. E. Briggs and R. N. Robertson . . . 265

Algae, recent migration from Red Sea into the Mediterranean—A. A. Aleem . . . 88

Ampoule effect, in the basidium—E. J. H. Corner . . . 22

Analysis of plant material, by polarographic methods—J. P. R. Riches . . . 1

Apparatus for extraction of gas from small amounts of plant sap or tissue—W. G. Burton and W. T. Spragg . . . 17

Area, of leaves of *Ipomaea*, in relation to position on the shoot—Eric Ashby . . . 177

Asterionella formosa Hass., Fluctuations in numbers in relation to fungal epidemics—Hilda M. Canter and J. W. G. Lund . . . 238

Basidium, Studies in the, I. The ampoule effect—E. J. H. Corner . . . 22

Berwickshire, Late-glacial deposits in—G. F. Mitchell . . . 262

Cell division, in the desmid *Cosmarium meneghinii*—J. M. Thoday . . . 292

Cell size and cell number of leaves of *Ipomaea*, in relation to position on the shoot—Eric Ashby . . . 177

Chromatophores, loss of, in *Euglena gracilis*—E. G. Pringsheim and R. Hovasse . . . 51

Clearing, A rapid technique of wide application—Kenneth R. Sporne . . . 290

Climatic rhythms, Von Post's work on—Verona M. Conway . . . 220

Cosmarium meneghinii, Cell division in—J. M. Thoday . . . 292

Coumarin, Studies in phytostatic action of—L. J. Audus . . . 196

Cytology of chromatophores in *Euglena gracilis*—R. Hovasse . . . 68

Diffusion and absorption in disks of plant tissue—G. E. Briggs and R. N. Robertson . . . 265

Ecology and taxonomy of Primrose and Oxlip (*Primula vulgaris* Huds. and *P. elatior* Schreb.)—D. H. Valentine . . . 111

Ecotypes of *Melandrium dioicum* (L. emend.) Coss. & Germ.—H. G. Baker . . . 131

Euglena gracilis, Loss of chromatophores in—E. G. Pringsheim and R. Hovasse . . . 51

Extraction of gas from small amounts of plant sap or tissue, Apparatus for—W. G. Burton and W. T. Spragg . . . 17

Floral symmetry, Nature and range of variation in, in *Potentilla erecta* (L.) Hampe—A. G. Tansley . . . 95

PAGE

Fungal epidemics, Fluctuations in numbers of *Asterionella formosa* Hass. in relation to—Hilda M. Canter and J. W. G. Lund 238

Gas extraction from small amounts of plant sap or tissue—W. G. Burton and W. T. Spragg 17

Inhibitions, root-growth, Reversibility of—L. J. Audus 196

Ipomaea, Area, cell size and cell number of leaves of, in relation to position on the shoot—Eric Ashby 177

Kalanchoë, Effect on stomatal behaviour of detaching leaves of—Husein Saïd and M. K. Tolba 284

Kalanchoë, Effect of stem-ringing on stomatal rhythm in leaves of—Husein Saïd . 288

Late-glacial deposits in Berwickshire—G. F. Mitchell 262

Leaf-shape, An essay on—Eric Ashby 153

Melandrium dioicum (L. emend.) Coss. & Germ., Ecotypes of—H. G. Baker . . 131

Mesembryanthemum, Effect on stomatal behaviour of detaching leaves of—Husein Saïd and M. K. Tolba 284

Migration of certain Indo-Pacific algae from Red Sea to Mediterranean—A. A. Aleem 88

Morphogenesis of leaves. I.—Eric Ashby 153

Morphogenesis of leaves. II.—Eric Ashby 177

Phytostatic action of 2:4-dichlorophenoxyacetic acid and coumarin—L. J. Audus . 196

Plankton parasites, studies on. I.—Hilda M. Canter and J. W. G. Lund . . . 238

Polarographic methods of plant analysis—J. P. R. Riches 1

Potentilla erecta (L.) Hampe, Nature and range of variation in floral symmetry of—A. G. Tansley 95

Primula vulgaris Huds. and *P. elatior* Schreb., Ecology and Taxonomy of—D. H. Valentine 111

Stem-ringing, Effect on daily march of stomatal rhythm in leaves of *Kalanchoë*—Husein Saïd 288

Stomatal behaviour, Effect of detaching leaves of *Kalanchoë* and *Mesembryanthemum* at different times of day on—Husein Saïd and M. K. Tolba 284

Stomatal rhythm, Effect of stem-ringing on, in leaves of *Kalanchoë*—Husein Saïd . 288

Taxonomy, Ecology and, of *Primula vulgaris* Huds. and *P. elatior* Schreb.—D. H. Valentine 111

PAGE

REVIEWS:

Den Skogliga Rasforskningen och Praktiken (Bertil Lindquist)—S. M. Walters . 148

Die Goldhaferwiese der Schweiz (Franz Marschall)—J. F. Hope-Simpson . . . 150

Heredity and Variation in Micro-organisms (Cold Spring Harbour Symposia on Quantitative Biology, Vol. XI)—J. M. Thoday 295

Hormones and Horticulture (George S. Avery and Elizabeth Bindloss Johnson, with the collaboration of Ruth M. Addoms and Betty F. Thomson)—L. J. Audus . 298

Introduction to Carbohydrate Biochemistry (D. J. Bell)—W. O. James 297

North American Species of *Mycena* (Alexander H. Smith)—J. L. Harley 151

Plant Physiology (Meirion Thomas)—E. W. Yemm 298

Plant Viruses (Kenneth M. Smith)—T. E. T. Bond 300

The Chemical Activities of Bacteria (E. F. Gale)—G. Metcalfe 294

The Genetics of Garden Plants (M. B. Crane and W. J. C. Lawrence)—H. L. K. Whitehouse 299

The genus *Crepis*, Parts I and II (E. B. Babcock)—W. B. Turrill 146

The Living Plant (I. V. Newman)—H. Godwin 297

The Nature and Prevention of the Cereal Rusts as exemplified in the Leaf Rust of Wheat (K. Starr Chester)—T. E. T. Bond 147

The Royal Expedition to New Spain (H. W. Rickett)—J. L. Gilmour 296

JUNE 1948 VOL. 47, NO. 1

AN INTRODUCTION TO POLAROGRAPHIC METHODS AND THEIR APPLICATION TO THE ANALYSIS OF PLANT MATERIAL

BY J. P. R. RICHES
Botany School, University of Cambridge

(With 9 figures in the text)

It is little more than 20 years since Heyrovsky (1924) introduced the polarograph for recording automatically the curves between current and voltage obtained during the course of an electrolysis when the potential difference between the electrodes was gradually increased (Fig. 1). To-day, thanks to the pioneer work of Heyrovsky and his school in Czechoslovakia, and, more recently, that of Kolthoff in America, the principles of polarography are well understood, and the instrument is established among workers using microchemical techniques. The monograph by Kolthoff & Lingane (1941) gives an excellent introduction to the theory and practice of polarography, together with details of specific applications which had appeared in the literature up to the end of 1940.

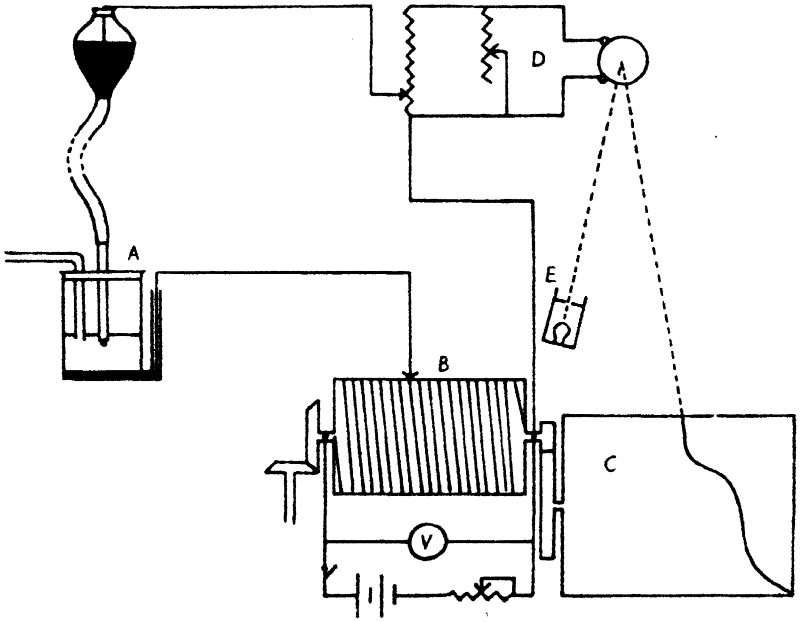

Fig. 1. The polarograph of Heyrovsky & Shikata. *A*, Electrolysis vessel containing test solution; *B*, rotating drum carrying potentiometer wire; *C*, cylinder coupled to drum carrying photographic paper; *D*, sensitive galvanometer with Ayrton shunt; *E*, galvanometer lamp.

When an electric current passes through a solution as the result of applying an E.M.F. across the electrodes, all the ions present in solution play a part in carrying the current, the contribution of each type depending on its concentration and mobility. But the ions discharged at the electrodes will be determined solely by their various deposition potentials, which for the various types of ions will follow a sequence corresponding closely to the well-known electrochemical series (noble metals being deposited at low potentials and alkali metals at high potentials). The deposition potential of a cation gives a measure of the energy required to remove the solvated ion from solution; its value is constant for any particular electrode in the absence of complex formation. A complex ion, however, such as cuprammonium, $Cu(NH_3)_4^{++}$, must be dissociated before electro-reduction of the free ion to the metal can take place; the deposition potentials of cations from solutions in which complexes are formed are usually more negative than those where the cation is merely solvated, since more energy is required to break down the complex than the solvated ion. This principle is useful in the separation of cations by electrolytic methods. For instance, copper and bismuth, whose deposition potentials in simple solution are close together, may be separated quantitatively if cyanide is added to the solution. (Cyanide forms a complex with copper but not with bismuth.)

In the polarographic cell, the *anode* is either a standard half cell, or the mercury layer which collects at the bottom of the electrolysis vessel. This layer, depolarized by the insoluble mercurous salt which forms in the presence of the fixed concentration of anion in the electrolyte, remains at approximately constant potential in the same way as the mercury of the calomel electrode. The precise potential of the mercury pool depends, however, on the types and concentrations of the anions present in solution, and, for precise work, an external calomel electrode is to be preferred. Majer (1937) has shown that the area of the mercury pool may be reduced to 1 sq.cm. without altering the current voltage curves appreciably. When the potential difference between the electrodes is increased, therefore, the change of potential is reflected mainly at the cathode, providing the fall of potential in the external circuit and in the solution is small. This is ensured in polarographic analysis by including in the test solution a high concentration of a supporting electrolyte whose cations are themselves not deposited in the potential range examined.

The *cathode* of the polarographic cell is a small mercury drop issuing from a capillary tube immersed in the test solution. The drop grows rapidly and finally falls from the capillary tip, being replaced there by another drop. The dimensions of the capillary and the head of mercury are adjusted to give a drop time of about 3 sec. The dropping mercury electrode has the advantage of presenting a constantly renewed clean spherical surface to the solution, so that the current is reproducible and independent of the previous course of analysis. Mercury provides a relatively inert electrode material with a high hydrogen over-potential. Moreover, in the presence of a high concentration of supporting electrolyte, the maximal rate of reaction at the drop surface is determined by the rate at which reducible substances can diffuse towards the drop (the rate of electrode processes being usually much greater than the diffusion rates). Thus, when sufficient potential is applied to cause any particular substance to react at the drop surface, the current does not increase indefinitely, but rises to a limiting value known as the *diffusion current*, determined by the concentration in the bulk solution and the rate of diffusion of the electro-reducible substance towards the drop. A well-marked 'step' appears in the current-voltage curve, and there is no further increase of current with increasing potential until the deposition potential of the next electro-reducible substance is reached.*

In a striking series of microphotographs, Antweiler (1938) showed that, under conditions favourable for polarographic analysis, a well-marked diffusion layer is built up around the drop surface; when the diffusion current is reached, the concentration of the electro-reducible substance falls from its value in the bulk of the solution down to zero concentration within a zone of thickness 0·05 mm. adjacent to the mercury surface (Fig. 2). If for any reason this diffusion layer is disturbed, by temperature or density gradients or electro-osmotic streaming, the true diffusion current is not obtained. It is customary in polarographic analysis to include in the supporting electrolyte a small amount of some substance (gelatin, methyl cellulose), which is adsorbed at the drop surface, and which suppresses irregularities in the current-voltage curve arising from disturbance of the diffusion layer. Care is required, however, in the choice of maximum suppressor, since it is known that some large molecules distort the polarographic step or reduce the diffusion current (Rayman, 1931; Wiesner, 1947).

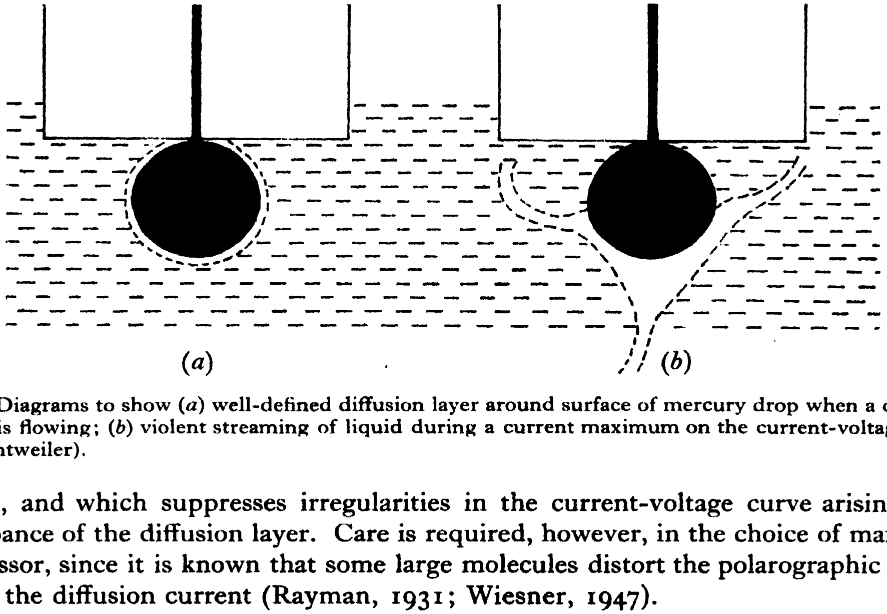

Fig. 2. Diagrams to show (*a*) well-defined diffusion layer around surface of mercury drop when a diffusion current is flowing; (*b*) violent streaming of liquid during a current maximum on the current-voltage curve (after Antweiler).

If, in the test solution, there are several cations reducible at the mercury cathode, the reductions will take place in turn as the potential is increased, and the polarogram then assumes a step-like appearance (Fig. 5). When the deposition is reversible, the metal being soluble in mercury, a symmetrical S-shaped curve is obtained, whose point of inflexion occurs at the ***half-wave potential*** ($E_{\frac{1}{2}}$), the current having risen to half its maximal value (Fig. 4).

The relation between potential and current for the reversible reduction of a simple metal ion to the free metal soluble in mercury is given by:

$$E = E_{\frac{1}{2}} - \frac{RT}{nF} \ln \frac{i}{i_D - i}, \tag{1}$$

---

* The amount of electro-reducible substance reacting at the tiny dropping mercury cathode will be small, and the bulk concentration of the electolysed solution will not diminish appreciably with time. When a mercury drop breaks away from the capillary, it carries with it the reduced product, which may accumulate in the solution or in the mercury layer at the bottom of the electrolysis cell. If this layer is charged positively with respect to the deposition potential, and if the reduction reaction in question is reversible, the substance may re-enter the solution in the unreduced form.

where E = average potential of cathode with reference to standard calomel electrode,
$E_{\frac{1}{2}}$ = half-wave potential,
i_D = diffusion current,
i = current at any point in polarographic step,
$\boldsymbol{R}$ = gas constant,
T = absolute temperature,
$\boldsymbol{F}$ = Faraday (96,500 coulombs).

Hence the plot of E against $\log i/(i_D - i)$ gives a straight line whose slope depends on the value of n, the number of electrons concerned in the electro-reduction. Similar relations may be derived for other types of drop reaction.

Heyrovsky & Ilkovic (1935) have shown that the half-wave potential for reversible processes is independent of the concentration of the reducible substance and also of the capillary constants. Generally, for a metal cation, it differs somewhat from the standard electrode potential of the metal (Lingane, 1939), the exact difference depending on the affinity of the metal for mercury and the kinetics of the diffusion processes in the solution and in the mercury drop.

From fundamental physical laws applied to an expanding spherical surface, Ilkovic (1938) and MacGillavry & Rideal (1937) have established the following expression for the current flowing at any instant during life of drop:

$$i = knD^{\frac{1}{2}}(C - C_0)\, m^{\frac{2}{3}} t^{\frac{1}{6}},^{*} \qquad (2)$$

where i = current flowing at any instant,
k = a numerical constant $\left[4\left(\frac{7\pi}{3}\right)^{\frac{1}{2}}\left(\frac{3}{4\pi\rho}\right)^{\frac{2}{3}}\boldsymbol{F}\right]$,
n = number of electrons concerned in drop reaction,
D = diffusivity constant of electro-reducible substance,
m = mass of mercury flowing from capillary per unit time,
t = time during life of drop.

If D, m, and t are measured in C.G.S. units, the bulk concentration C in moles per ml. and the current in amperes, k has the value 0·706. This factor includes the Faraday ($\boldsymbol{F}$) in coulombs and the density of mercury (ρ) in g. per ml.

During the formation of an individual drop at the dropping electrode, therefore, the current-time curve will be a sixth-order parabola (Fig. 3), rising to the value given by equation (2). In routine polarographic work, it is usually more convenient to measure the average current during the life of the drop, rather than the maximum value. When a damped galvanometer is used to record the current, the instrument is unable to follow the complete current time curves of individual drops (Fig. 3), but measures instead the average current during the life of the drop (which is theoretically six-sevenths of the value of the maximum current.[†] Thus $\bar{i} = 0{\cdot}605\, nD^{\frac{1}{2}}Cm^{\frac{2}{3}}\tau^{\frac{1}{6}}$.

---

[*] This equation is derived from the simple concept $i = dq/dt = nFA\,(dN/Adt)$. The flux dN/Adt is calculated or the dropping mercury electrode by applying Fick's law, $dN/dt = DA\,(\partial C/\partial r)_r$, to an expanding sphere, where the area of the diffusion field increases with time.

[†] The average current passing during the life of one drop will be equal to the total amount of electricity conerned in the drop reaction during the time interval τ (the drop time), divided by the time τ. From the Ilkovic quation, we have

$$\text{average current} = \frac{1}{\tau}\int_0^{\tau} knD^{\frac{1}{2}}Cm^{\frac{2}{3}}t^{\frac{1}{6}}\,dt = \frac{1}{\tau}\int_0^{\tau} k't^{\frac{1}{6}}\,dt$$

$$= \frac{1}{\tau}\left(\frac{6}{7}k'\tau^{\frac{7}{6}}\right) = \frac{6}{7}k'\tau^{\frac{1}{6}} = \frac{6}{7}\,(\text{maximum current}) \text{ when } \rho,\ D,\ C,\ m \text{ are constant.}$$

The Ilkovic equation has been tested experimentally (Kolthoff & Lingane, 1941, chap. IV), in the case of several reactions occurring at the dropping mercury electrode, and appears to be closely obeyed. Lingane & Loveridge (1946) have reported slight departures with rapidly flowing capillaries, where of course the simple assumptions upon which the Ilkovic equation is based will no longer strictly hold.

In testing the Ilkovic equation, the factors C, m and t may be measured experimentally, and the appropriate value of n obtained from a suitable i/E plot. The value of D, the diffusivity coefficient of the electro-reducible substance, is rarely available in the literature, and must be calculated in the case of ions from the mobility at infinite dilution, or, for uncharged molecules, from the Stokes-Einstein diffusion equation. These methods of calculation are valid only for ideal ions and molecules; they do not give exact diffusion coefficients under the conditions of polarographic test. However, in tests of the Ilkovic equation using thallous, lead, iodate and chromate ions, the observed and calculated diffusion currents agree within the limits of experimental error. It is now often assumed that the Ilkovic equation is obeyed, and may therefore be used to calculate diffusion coefficients in new electro-reductions, or, alternatively, the number of electrons concerned in the reaction at the drop surface, e.g. in the reduction of organic molecules (Lingane, 1945 *a*, *b*).

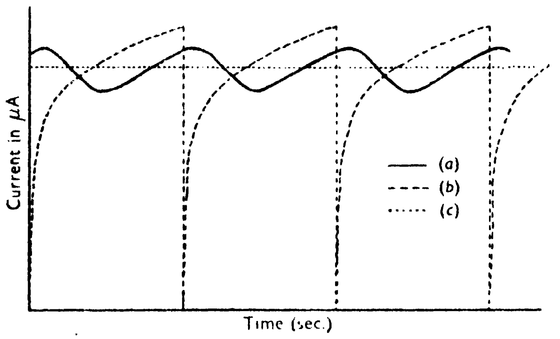

Fig. 3. (*a*) True current-time curves with dropping mercury cathode; (*b*) oscillations of the damped galvanometer; (*c*) theoretical value of average current.

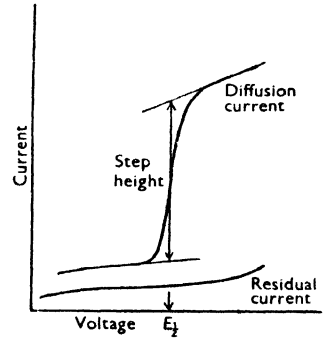

Fig. 4. Typical 'step' on current-voltage curve. (2×10^{-4}M zinc in acetate-thiocyanate. Temperature 16° C.)

The diffusion current then is directly proportional to the concentration of the electro-reducible substance, providing all the other factors are held constant. Thus, in any one supporting electrolyte at a fixed temperature, using the same capillary electrode, the magnitude of the diffusion current ('step height') gives a measure of the concentration, and the nature of the electro-reducible substance is deduced from the half-wave potential (Fig. 4). If there are present in the solution two reducible substances with deposition potentials within 0·2 V. of each other, the two reduction steps coalesce, and interpretation is difficult. In such cases it is best to use another supporting electrolyte in which the deposition potentials are further apart; alternatively, one of the substances may be removed by extraction or precipitation, and the resulting reduction in the step height measured.

Even in the absence of electro-reducible substances in the test solution, a small ***residual current*** flows through the polarographic cell, which must be subtracted from any apparent diffusion current in order to obtain the true diffusion current (Fig. 4). This residual current is caused by the charging of the double layer at the drop surface (condenser current), together with the current corresponding to any reduction occurring of traces of reducible impurities (Lingane & Loveridge, 1944). The slope of the residual current often masks the smaller steps on the current-voltage curve. Accordingly, Ilkovic & Semerano (1932) introduced into the polarographic circuit resistances designed to compensate the residual current, by sending a current of equal magnitude in the reverse direction through the galvanometer.

In any polarographic estimation, a calibration curve may be constructed between the step heights obtained for a series of concentrations of the electro-reducible substance (Fig. 6). If a linear relationship is established, the factor i_D/C may be used in subsequent work. Instead of the factor i_D/C, Lingane (1943) has introduced the notion of '*diffusion current constant*' $\left(\frac{i_D}{Cm^{2/3}t^{1/6}}\right)$, which is independent of the capillary constants and depends only on the nature of the electro-reducible substances (providing the conditions are maintained constant and the Ilkovic equation is obeyed). By the use of such factors, it should be possible to convert step heights directly to concentrations for any capillary, once the standard data have been collected.

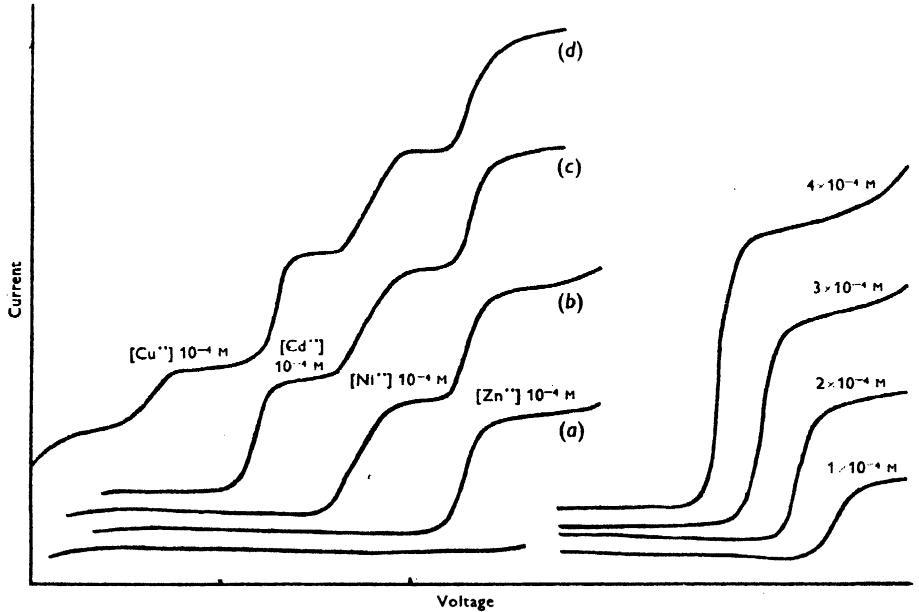

Fig. 5 Fig. 6

Fig. 5. Current-voltage curves showing steps of (*a*) zinc; (*b*) nickel and zinc; (*c*) cadmium, nickel and zinc; (*d*) copper, cadmium, nickel and zinc, solutions at concentrations each approximately 10^{-4}M. Supporting electrolyte ammoniacal buffer; temperature 18° C.

Fig. 6. 'Step' height increasing with increasing concentrations of zinc in ammoniacal buffer. Temperature 20° C.

Since the magnitude of the diffusion current depends on the number of electrons involved in the reaction at the drop surface, a greater polarographic sensitivity is to be expected for reactions involving the transfer of many electrons. (At the same time, the slope of the current-voltage curve will be steeper, providing the reaction is reversible.)

Monovalent cations unite with one electron, divalent with two. Many oxyacids are reducible at the dropping mercury electrode. Bromate and iodate anions are reduced in buffered acid medium with the uptake of six electrons (Orlemann & Kolthoff, 1942),

$$IO_3' + 6\epsilon + 6H^+ \rightarrow I' + 3H_2O,$$

the half-wave potential becoming more negative with decreasing hydrogen ion concentration of the medium. Permanganate and chromate ions are reduced in several steps corresponding to successively lower levels of oxidation, the diffusion current of intermediary steps being sometimes well defined. The magnitude of the diffusion currents gives some idea of the stages of electro-reduction (Astle & Cropper, 1943), and confirmatory evidence comes from the actual isolations of reduced products, using a large cathode maintained at constant potential. Thus Lingane (1945*b*) foretold the substance formed when picric acid is reduced. The polarographic curves suggested a reduction reaction involving seventeen electrons per molecule, corresponding to the reduction product di-(4-hydroxy-3, 5-diaminophenyl) hydrazine. A great many organic substances are reducible at the dropping mercury electrode, and give well-defined diffusion currents, but the reactions are rarely reversible, and are usually dependent on the nature of the supporting electrolyte, and frequently on the hydrogen ion concentration (Müller, 1939; Pasternak & v. Halben, 1946). However, the polarographic waves may often be used for analytical purposes, providing the test solution is free from other reducible substances which would interfere with the estimation (Lingane & Davis, 1941; Beaver & Kauwitz, 1944).

Although the net changes at the drop surface have been established in many reactions, the mechanism of reduction has rarely been elucidated. This is hardly surprising when it is considered that even the mechanism of hydrogen deposition is still in dispute. In the reduction of aromatic aldehydes, Baker, Davies & Hemming (1940) have suggested the recession of an electron from the attached group, and subsequent reaction with hydrogen ion from the solution (cf. final footnote).

Polarographic steps are obtained when the mercury drop is made the *anode* of the electrolysis cell, in solutions containing ions which form insoluble salts or complex ions with mercury (e.g. chloride, hydroxide, sulphite).

Molecular oxygen is reduced at the dropping mercury cathode, first to hydrogen peroxide; and, at higher negative potentials, to water. Consequently, the oxygen must be removed from a test solution if the oxygen steps interfere with the estimations. This is done by sweeping out the oxygen from the solution with a current of pure hydrogen or nitrogen gas, or, if the test solution is alkaline, by adding sulphite.

By means of the polarograph, concentrations of electro-reducible substances as low as 10^{-5}M may be estimated with an accuracy of 5%, and the volume of the test solution need be no more than 1 ml. Majer has described microcells in which the estimation may be performed with even smaller volumes of solution.

Satisfactory current-voltage curves may be obtained without a recording polarograph, using a simple circuit assembled from standard laboratory apparatus, a voltage divider and a sensitive galvanometer with an Ayrton shunt, or a microammeter with a condenser across its terminals to damp the oscillations. Such a circuit is adequate for many purposes, and has advantages where the changes in concentration of a single substance are being followed by continuous record (Manning, 1940).* In the recording polarographs now available, the current is measured either by a sensitive galvanometer which reflects the light beam back to a photographic paper carried by a rotating drum, as in the original Heyrovsky machine (Fig. 1), or directly by means of an automatic potentiometer recorder with a pen and ink trace. There have been attempts to use a cathode ray oscillograph for recording current-voltage curves (Müller, Garman, Droz & Petras, 1938; Nickelson, 1946, Randles, 1947). In both the pen-recording and the cathode ray polarograph, the recording instrument is undamped, and a complete current record is possible, including the changes during the growth of each drop. There are advantages in measuring the maximum rather than the average diffusion current, both in sensitivity (maximum current $= \frac{7}{6} \times$ average current) and in accuracy (the true average current being somewhat difficult to estimate from the oscillations of a damped galvanometer).

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* The latest American models permit this type of continuous recording.

Kolthoff (1942) has discussed the technique of polarography in some detail. Many points have already been considered—the use of supporting electrolytes, the necessity for a constant dropping rate, the removal of oxygen and reducible impurities, and the correction for residual current. Capillaries may be hand-drawn from pyrex capillary tubing, or obtained directly from the manufacturers. An internal diameter of 0·05 mm. gives a convenient drop rate with the level in the mercury reservoir 50 cm. above the capillary tip. It is extremely important that the temperature should be controlled in polarographic analysis. Temperature changes affect the factors ρ, D, C, m, t, the relative importance being predictable from the Ilkovic equation.

The variation of the diffusion coefficient with temperature is much greater than any of the others, and largely determines the temperature coefficient of the diffusion current. The observed temperature coefficient (1·3–2·3% per °C.) is in good agreement with theory, and corresponds to a variation of diffusion current of 1% for a change of temperature (±0·5° C.).

It is often useful in routine analysis to include a known concentration of *thallium* ions in the supporting electrolyte, providing the thallium wave does not interfere with any other. From the thallium step height in particular polarograms, a correction may be applied for any slight temperature changes in a series of estimations, and, since the half-wave potential of thallium is constant for many supporting electrolytes, this value may be used to fix a point on the potential axis without resort to an external standard half cell.

Kolthoff and his co-workers (1940) have shown that the dropping mercury electrode may be used as an indicator of titration end-points. Just as the sharp break in the conductivity curve has been used in conductometric methods, so the end-point can be estimated by the sudden increase or decrease of diffusion current when a constant potential is employed.

THE ANALYSIS OF PLANT MATERIAL

It is clear that the polarographic method offers a new and extremely sensitive aid in the *analysis of plant material.* If we consider first the inorganic components, there are present, in addition to the major elements calcium, potassium, magnesium and phosphorus, varying quantities of many other elements. Thus, if 1 g. of plant material is dissolved to give a test solution of volume 10 ml., the various cations will be present in approximately the concentrations shown in Table 1.

For the elements present only in traces, it has in the past been necessary to use large samples of plant material or to concentrate the test solution to small volumes. Since analytical reagents often contain trace impurities, and contamination may also arise from the glassware or the distilled water, the accurate estimation of trace elements calls for the greatest care.

It is by no means easy to prepare from plant material a solution suitable for quantitative estimations of inorganic constituents. Solutions are usually prepared either by digesting the plant material with strong mineral acids in the presence of an oxidizing agent (Eden & Green, 1940; Piper, 1942), or by ashing in a muffle furnace and dissolving the ash in hydrochloric acid. There are difficulties in the use of each of these procedures if a solution is required suitable for polarographic analysis.

Ashing leads to appreciable loss of the more volatile components, e.g. copper, zinc, even in the presence of a basic substance such as magnesium nitrate (Briggs, 1941). Piper (1942) has shown, moreover, that the surface of the ashing vessel may adsorb trace elements. Further, the test solution once prepared must be kept at a pH greater than 5, otherwise a precipitate of aluminium hydroxide and calcium phosphate forms, on whose surface the heavy metals are adsorbed. This loss of cations from solution by adsorption on the surface of precipitates was an uncontrolled source of error in early work. The percentage error increases with diminishing size of sample.

Table 1

	Fraction of dry material (%)	Concentration (g.equiv./l.)
Calcium	2	0·1
Potassium	2	0·05
Magnesium	0·3	0·02
Phosphorus	0·3	0·03
Iron Manganese Sulphur Zinc	0·01	0·0004
Copper Molybdenum	0·001	0·00004
Nickel Cobalt	0·0001	0·000004

These losses are all avoided if the plant material is *digested with strong acids*, but the remaining solution is of high total ionic concentration, since it contains, in addition to the components of the plant material, an unavoidable excess of non-volatile reagents. Such a solution is unsuitable for immediate polarographic analysis (Fig. 7*a*), although specific colour tests may sometimes be applied. As in the majority of colour tests, therefore, it is necessary to isolate specific elements before estimation; this is accomplished either by precipitation or by extraction. The introduction of organic reagents has led to the discovery of many rapid and specific preliminary chemical separations.

Despite the limitations of current-voltage curves when untreated plant digests are taken, the rapidity of the method often recommends it in certain applications (Fig. 7*b*). Reed & Cummings (1940) have used a solution of plant ash for determining the *zinc* in plant materials; in a supporting electrolyte containing acetate and thiocyanate ions, there is no precipitate formation, the trace elements remaining completely in solution. In this supporting electrolyte, the only cation with half-wave potential close enough to that of zinc to give coalescence of wave is cobalt, which in most plant materials is present in extremely small quantities. A solution of plant ash is, however, unsuitable for the estimation of *copper*, since recoveries are invariably low; Reed & Cummings (1941) have therefore recommended an acid digestion method; the copper step occurs early in the polarogram, and there is little interference from other reducible substances.

The addition to a test solution of *specific reagents* for eliminating the waves of individual elements in the polarogram is not feasible; for complete removal an excess of reagent is necessary; most reagents themselves are reducible and at high concentrations distort the current-voltage curves.

In most polarographic methods for the inorganic analysis of plant material, therefore, a preliminary chemical separation is advisable. For the estimation of *zinc*, Walkley (1942) recommends the reagent dithizone, and the same reagent or its analogue di-β-naphthylthiocarbazone, has been used in the estimation of *cadmium* (Cholak & Hubbard, 1944).

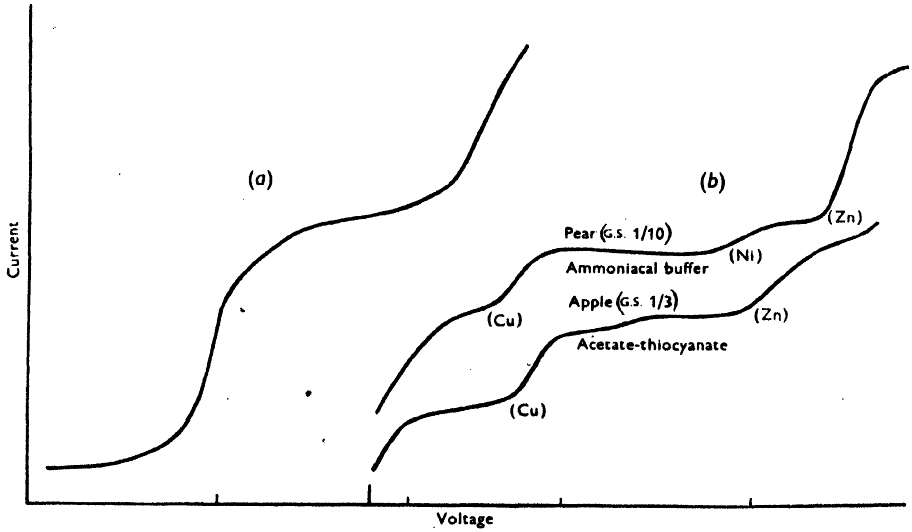

Fig. 7. (*a*) Current-voltage curve for a plant digest, showing only poorly defined steps; supporting electrolyte: acetate-thiocyanate; (*b*) current-voltage curves for solutions of plant ash dissolved in dilute acid.

Molybdenum may be extracted by means of α-benzoinoxime (Nichols & Rogers, 1944), and *nickel* with dimethylglyoxime; *magnesium* with 8-hydroxyquinoline (Stone & Furman, 1944) and *sodium* with zinc uranyl acetate (Carruthers, 1943, 1945). *Arsenic* is isolated by liberating gaseous arsine and adsorbing it in mercuric chloride solution (Bambuch, 1942).

Some of the methods described originally for colorimetric estimations may be readily converted to polarographic tests. Thus Cohn & Kolthoff (1943) have described a method for estimating *calcium* in plant material by measuring the reduction in step height of picrolonic acid, caused by the formation of a calcium picrolonate precipitate. The concentration of calcium is so high in a plant digest compared with other cations which, under the conditions of the test, form precipitates with picrolonic acid, that no correction is necessary. However, calcium picrolonate is appreciably soluble in the acetate buffer used, and a knowledge of the solubility product is necessary in interpreting the concentrations measured. As Moore, Stein & Bergmann (1942) have pointed out, low concentrations may often be estimated without quantitative precipitation, provided that the solubility product in the test solution is known.

A similar indirect measure of the *potassium* may be obtained by measuring the reduction of height of the dipicrylamine step after the precipitation of the potassium salt (Kolthoff & Bendix, 1939).

There are a few organic reagents which form complexes or precipitates with a range of cations. For instance, 8-hydroxyquinoline precipitates from solution the metals magnesium, aluminium, zinc, copper, cobalt, nickel and molybdenum. Scott & Mitchell (1943) have used this reagent, alone or in mixture with cupferron, to estimate trace elements in soils and plants. The traces are precipitated together and the precipitate after ignition examined spectroscopically. The method is admirable if 10 g. samples are available, but, with smaller samples, precipitation is incomplete.

It was hoped that the reagent dithizone might be used to isolate simultaneously a number of elements from a plant digest. Dithizone forms, with many metals, complexes which are soluble in chloroform or carbon tetrachloride. By the use of a solution of dithizone in chloroform, the metals may be extracted from an aqueous solution. The addition to the separated chloroform layer of dilute hydrochloric acid breaks down the dithizone complexes, and returns the metals quantitatively to the aqueous phase, free from other components of the digest (and often in higher concentration than in the original mixture). Although it is possible to define conditions where one particular metal is isolated quantitatively from a mixed solution (Wichmann, 1939; Kolthoff & Sandall, 1941), there appears to be no single set of conditions allowing the simultaneous extraction of several metals quantitatively. There is a pH range peculiar to each element, within which the metal complex is stable, and the ranges for different elements do not overlap sufficiently.

The investigation of reagents specific for the isolation of metals has in the past been largely empirical; there are available in the literature all too few data on solubility products and partition coefficients for these reactions.

Unless a method can be found for the simultaneous extraction of a number of trace elements from a plant digest, one of the great advantages of the polarographic method is wasted, namely, the possibility of *estimating several elements in a single polarogram*. Preliminary results suggest that *synthetic resins* may serve for this type of isolation.

There are now available in America a series of *synthetic resins* possessing powers of base exchange similar to those found in zeolites. Since the discovery of the adsorptive properties of synthetic resins by Adams & Holmes (1935), these substances have been widely used in purification techniques (Beaton & Furnas, 1941; Myers, 1943; McReady & Hassid 1944). The cation exchanger, Amberlite IR 100, a sulphonated phenol formaldehyde polymer, has a stable, flexible and porous structure, with a large active internal surface. A column containing the powdered resin gives satisfactory separation of cations from accompanying anions (Riches, 1946*c*). If the properties of the cations are sufficiently different, individual cations may be isolated in successive fractions of the effluent solution. Since the anions in the original solution pass through the column unadsorbed, it is possible to obtain the cations on elution in association with any desired anion. Thus, in a plant digest, the trace elements may be separated from phosphate ions, and this allows a subsequent estimation in neutral or alkaline solutions without the formation of phosphate precipitates. If the trace elements are present in a solution of high total ionic concentration, however, they are not retained by the column, but are 'chased down' by the high cation concentration. It is evident that conditions for isolating trace elements from an acid digest will need to be carefully defined.

Once the various ions are isolated in fractions free from interfering substances, it becomes possible to choose the most suitable supporting electrolyte for quantitative work. Riches (1946*a*) has shown that, if phosphate and aluminium ions are removed from the solution, the trace elements—copper, cadmium, nickel, zinc and manganese—may be estimated simultaneously in an ammoniacal buffer containing lithium ions. This supporting electrolyte has several advantages:

(*a*) It gives well-defined and well-separated reduction steps on the current-voltage curve.

(*b*) Sulphite may be used to remove the oxygen from the test solution.

(*c*) The diffusion currents remain well defined with increasing concentration of the buffer, even at concentrations higher than molar.

(*d*) The diffusion currents remain well defined in the presence of considerable concentrations of various cations. Thus the potassium and calcium ions from a plant digest need not be removed if this supporting electrolyte is used.

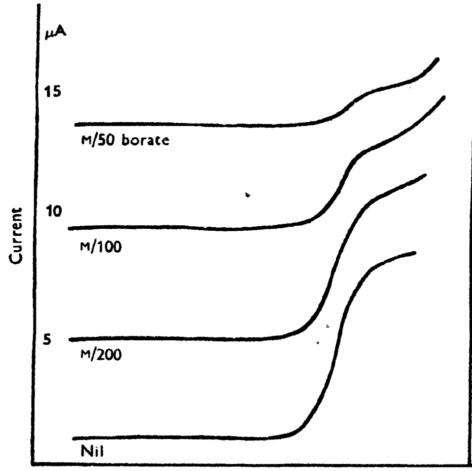

Fig. 8

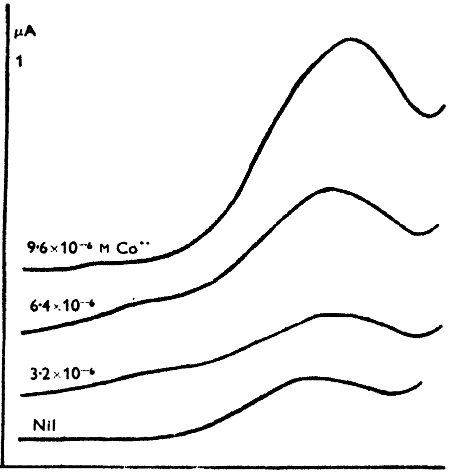

Fig. 9

Fig. 8. Reduction of 'step' height of phthalic acid ($2 \cdot 5 \times 10^{-3}$M) on addition of borate. Supporting electrolyte: acetate pH 4·1. Temperature 20° C.

Fig. 9. Catalytic waves obtained with cystine (2×10^{-6}M) in ammoniacal solutions, with increasing concentrations of cobalt. Temperature 20° C.

There have been few attempts hitherto to estimate anions in a plant digest polarographically, although many drop reactions have been described for anions in simpler solutions. If the various anions in a plant digest (phosphate, chloride, sulphate, silicate, molybdate, borate) can be isolated from interfering substances by the use of synthetic resin anion exchangers, polarographic estimation should be possible. Even the borate ion, which is notoriously difficult to estimate in low concentrations (Berger & Truog, 1939, 1943), can be determined indirectly by measuring the reduction of step height of a substance with which it forms a non-reducible complex (Fig. 8); cf. Pasternak, 1947.

Since *molecular oxygen* is reducible at the dropping mercury cathode, the concentration of dissolved gas is readily measured from a polarogram, in the absence of other substances reducible at low negative potentials. In this way, Karsten (1938) determined the dissolved oxygen in soil solutions, and Petering & Daniels (1938) the rate of oxygen uptake by *Chlorella pyrenoidosa*. Using a dropping electrode maintained at constant potential, Manning (1940) was able to arrange for continuous records to be taken of the oxygen dissolved in lake waters (cf. Ingols, 1942).

Stiles & Skelding (1940) have followed the uptake of manganous ions by slices of carrot tissue, by measuring at intervals the concentration of the external solution. Babicka (1934) also analysed external solutions to demonstrate the presence of substances diffusing from cut roots.

Although many organic substances in pure solutions are reducible at the dropping mercury electrode, and often give well-defined steps (Müller, 1939), the polarographic method has not replaced the well-tried methods already in use for the components of plant material. In extracts containing organic substances, there are usually present many reducible species besides those being estimated, and, unless a preliminary extraction is made, the steps on the polarogram are not precise enough for quantitative work. On the other hand, colour methods may sometimes be employed without preliminary extraction (e.g. phosphate). Occasionally the advantages lie with the polarographic method, however; for example, Gillam (1945) determined ascorbic acid in fruits and vegetables, by extracting with oxalic and metaphosphoric acids at pH 3·5, and recording the current-voltage curves for the coloured extract, using a dropping mercury electrode. Ketosteroids (Barnett, Henly & Morris, 1946) and components of the Vitamin B complex (Lingane & Davis, 1941) have been estimated by means of the polarograph. It may be possible to estimate adenine in hydrolysates of nucleic acids (Heath, 1946).

There have been numerous attempts to estimate amino-acids polarographically. Both proteins and amino-acids catalyse the deposition of hydrogen, producing characteristic maxima in the current-voltage curves. In the presence of cobaltous ions, there is a further catalytic effect, which Brdicka (1933) attributes to complex formation between SH groups and the cobalt ion, rendering the SH group more readily deformable. The hydrogen ion is split off at the interface, and is immediately replaced by hydrogen from the solvent. The remaining hydroxyl ion combines with an ammonium ion and the reformed SH group reacts again. Catalytic currents do not obey the Ilkovic equation (Müller & Davis, 1945), but the polarograms are reproducible, and serve for the estimation either of the catalyst or of minute traces of cobalt (Riches, 1947) (Fig. 9). In general, such methods are unsuitable with mixed solutions (Stern, Beach & Macy, 1939; Roberts, 1941). Catalytic currents are proportional, not to $m^{\frac{2}{3}}t^{\frac{1}{6}}$, but to $m^{\frac{2}{3}}t^{\frac{2}{3}}$, since they depend on the maximal surface area of the mercury drop (Müller & Davis, 1945). The special capillary with a constriction above the orifice (Riches, 1946*b*) may prove useful in this type of work.

Reactions catalysed by enzymes have been studied polarographically; for example, catalase and peroxidase (Brdicka & Tropp, 1937; Haurowitz, 1937; Walker, 1941), and the proteases of serum (Kotljar & Podrouzek, 1938). Another type of polarographic test of high sensitivity depends on the suppression of maxima in the current-voltage curves by substances which are adsorbed at the surface of the mercury drop. Hamamoto (1933) suggested a test of this type for alkaloids (cf. Kirkpatrick, 1946), and Ingram (1941) has estimated the protein content of brines by measuring the concentration of methylene blue necessary to suppress the catalytic wave.

It is hoped that this brief introduction to the polarographic method will suffice to illustrate its versatility, and to suggest new fields where the instrument might be used with advantage.*

The original experimental work mentioned in the section on analysis of plant material was carried out at East Malling Research Station, whilst working in the team financed by the Agricultural Research Council for research on the diagnosis and cure of mineral deficiencies in plants.

REFERENCES

ADAMS, B. A. & HOLMES, E. L. (1935). Adsorptive properties of synthetic resins. *J. Soc. Chem. Ind., Lond.*, **54**, 1T.

AMES, S. R. & DAWSON, C. R. (1945). Determination of copper in copper proteins. *Industr. Engng Chem.* (Anal. ed.), **17**, 249.

ANTWEILER, H. V. (1938). Methoden zur Beobachtung der Diffusionsschlicht bei der elektrolytischen Abscheidung. *Z. Elektrochem.* **44**, 719.

ASTLE, M. J. & CROPPER, W. P. (1943). Polarographic investigation of hydrogen bonding. *J. Amer. Chem. Soc.* **65**, 35, 2395.

BABICKA, J. (1934). Analysen der aus pflanzlichen Organen exosmodierenden Stoffe. *Beih. bot. Zbl.* **52**A, 485.

BAILEY, L. F. & McHARGUE, J. S. (1945). Ashing procedures in the determination of copper in plant material. *Plant Physiol.* **20**, 79.

BAKER, J. W., DAVIES, W. C. & HEMMING, M. L. (1940). Electrolytic reduction of carbonyl compounds. *J. Chem. Soc.* 692.

BAMBUCH, K. (1942). Polarographic determination of arsenic in biological material. *Industr. Engng Chem.* (Anal. ed.), **14**, 265; **16**, 339.

BARNETT, J., HENLY, A. A. & MORRIS, C. J. O. R. (1946). The polarographic estimation of steroid hormones. *Biochem. J.* **40**, 445, 450.

BEATON, R. H. & FURNAS, C. C. (1941). Concentration of dilute solutions of electrolytes by base exchange materials. *Industr. Engng Chem.* **33**, 1500.

BEAVER, J. J. & KAUWITZ, H. (1944). Interference with the polarographic determination of α-tocopherol. *J. Biol. Chem.* **152**, 363.

BERGER, K. C. & TRUOG, E. (1939). Boron determination in soils and plants. *Industr. Engng Chem.* (Anal. ed.), **11**, 540.

BERGER, K. C. & TRUOG, E. (1943). Boron tests and determination for soils and plants. *Soil Sci.* **57**, 25.

BIEBER, R. & TRUMPLER, G. (1947). Über die polarographische Reduktion der aliphatischen Aldehyde. *Helv. chim. Acta*, **30**, 706, 971, 1109.

BRDICKA, R. (1933). A new test for proteins. *Collection Czechoslov. Chem. Commun.* **5**, 112.

BRDICKA, R. (1947). Measurements of quantities concerning the adsorption of certain reducible compounds or their reduction products at the dropping mercury electrode. *Collection Czechoslov. Chem. Commun.* **12**, 522.

BRDICKA, R. & TROPP, C. (1937). Polarographische Untersuchungen über Blutfarbestoffe und ihre Derivate. *Biochem. Z.* **289**, 301.

BRDICKA, R. & WIESNER, K. (1947). Polarographic determination of the rate of the reaction between ferrohem and hydrogen peroxide. *Collection Czechoslov. Chem. Commun.* **12**, 39.

BRIGGS, C. H. (1941). Ash determination in cereals and other vegetable materials. *Industr. Engng Chem.* (Anal. ed.), **11**, 163.

CARRUTHERS, C. (1943). Ultramicromethod for sodium and microdetermination of magnesium. *Industr. Engng Chem.* (Anal. ed.), **15**, 70, 412.

CARRUTHERS, C. (1945). Microdetermination of copper. *Industr. Engng Chem.* (Anal. ed.), **17**, 398.

CHOLAK, J. & HUBBARD, D. M. (1944). Determination of cadmium in biological materials. *Industr. Engng Chem.* (Anal. ed.), **16**, 333.

CHOLAK, J., HUBBARD, D. M. & BURKEY, R. E. (1943). Determination of zinc in biological materials. *Industr. Engng Chem.* (Anal. ed.), **15**, 754.

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* There have been several interesting developments in the field of polarography since the time when this review was prepared. (cf. Heyrovsky, 1947). The introduction of oscillographic recording of potential-time curves and the use of kinetic analysis of the steps corresponding to irreversible reductions (Bieber & Trumpler, 1947) have made possible a deeper insight into the mechanism of reactions occurring at the drop surface. A comparison of polarographic behaviour of related organic substances (Keller, Hochweber & v. Halben, 1946) promises to throw further light on the effect of substituent groups in altering the reactivity of molecules. The more important references have been added to the bibliography.

Cohn, H. & Kolthoff, I. M. (1943). Determination of calcium by precipitation with picrolonic acid. *J. Biol. Chem.* **147**, 705.

Eden, A. & Green, H. H. (1940). Microdetermination of copper in biological material. *Biochem. J.* **34**, 1201.

Furman, N. H., Bricker, C. E. & Whitesell, E. B. (1942). Construction and operation of a polarograph. *Industr. Engng Chem.* (Anal. ed.), **14**, 333.

Gillam, W. S. (1945). Polarographic determination of vitamin C in fruits and vegetables. *Industr. Engng Chem.* (Anal. ed.), **17**, 217.

Goodwin, L. G. & Page, V. E. (1943). A study of the excretion of organic antimonials. *Biochem. J.* **37**, 198.

Hamamoto, E. (1933). The suppressive effect of certain alkaloids on maxima of current due to electroreduction of oxygen. *Collection Czechoslov. Chem. Commun.* **5**, 427.

Haurowitz, F. (1937). The catalase and peroxidase action of hemin. *Enzymologia*, **2**, 9.

Heath, J. C. (1946). Polarographic behaviour of adenine. *Nature, Lond.*, **158**, 23.

Heyrovsky, J. (1924). The processes at the dropping mercury cathode. *Trans. Faraday Soc.* **19**, 692.

Heyrovsky, J. (1947). The fundamental laws of polarography. *Analyst.* **72**, 229.

Heyrovsky, J. & Ilkovic, D. (1935). The absolute determination of reduction and depolarisation potentials. *Collection Czechoslov. Chem. Commun.* **7**, 198.

Ilkovic, D. (1938). The value of the diffusion current observed during electrolysis at the dropping mercury electrode. *J. Chem. Phys.* **35**, 129.

Ilkovic, D. & Semerano, G. (1932). Increased sensitivity by a compensation of current. *Collection Czechoslov. Chem. Commun.* **4**, 176.

Ingols, R. S. (1942).. Dissolved oxygen recordings with the dropping mercury electrode. *Industr. Engng Chem.* (Anal. ed.), **14**, 256.

Ingram, M. (1941). Analysis of brines. *J. Soc. Chem. Ind., Lond.*, **60**, 126.

Karsten, K. S. (1938). Root activity and oxygen requirement. *Amer. J. Bot.* **26**, 855.

Keller, H., Hochweber, M. & v. Halben, H. (1946). Polarographische und spektrographische Untersuchungen an organischen Halogenverbindungen. *Helv. chim. Acta*, **29**, 761.

Kirkpatrick, H. F. W. (1946). Polarographic study of alkaloids. *Quart J. Pharm.* **19**, 526.

Kolthoff, I. M. (1942). Factors to be considered in quantitative polarography. *Industr. Engng Chem.* (Anal. ed.), **14**, 195.

Kolthoff, I. M. & Bendix, G. H. (1939). Determination of potassium with dipicrylamine. *Industr. Engng Chem.* (Anal. ed.), **11**, 94; **12**, 731.

Kolthoff, I. M. & Lingane, J. J. (1941). *Polarography*. New York: Interscience Publishers Inc.

Kolthoff, I. M. & Sandall, E. B. (1941). A quantitative expression for the extractibility of metals in the form of dithizonates. *J. Amer. Chem. Soc.* **63**, 1906.

Kolthoff, I. M. & co-workers (1940). Amperometric titrations. *J. Amer. Chem. Soc.* **61**, 3332, 3402; **62**, 211, 3172, 3332.

Kotljar, A. M. & Podrouzek, V. (1938). Polarographic proof of proteolysis in diagnosis with enzyme reactions. *Nature, Lond.*, **142**, 872.

Laitinen, H. A. & Wawsonee, S. (1942). Reduction of unsaturated hydrocarbons. *J. Amer. Chem. Soc.* **64**, 1765.

Lingane, J. J. (1939). Thermodynamic significance of polarographic half-wave potentials. *J. Amer. Chem. Soc.* **61**, 2099.

Lingane, J. J. (1943). Systematic polarographic metal analysis. *Industr. Engng Chem.* (Anal. ed.), **15**, 583.

Lingane, J. J. (1945*a*). Automatic apparatus for electrolysis at controlled potentials. *Industr. Engng Chem.* (Anal. ed.), **17**, 332.

Lingane, J. J. (1945*b*). Coulometric analysis. *J. Amer. Chem. Soc.* **67**, 1916.

Lingane, J. J. & Davis, O. L. (1941). Polarographic determination of riboflavin. *J. Biol. Chem.* **137**, 567.

Lingane, J. J. & Loveridge, B. A. (1944). Influence of capillary characteristics on diffusion current and residual current. *J. Amer. Chem. Soc.* **66**, 1425.

Lingane, J. J. & Loveridge, B. A. (1946). Empirical modification of the Ilkovic equation. *J. Amer. Chem. Soc.* **68**, 395.

Lupton, J. M. & Lynch, C. C. (1944). Polarographic estimation of carbonyl compounds. *J. Amer. Chem. Soc.* **66**, 697.

Lyman, C. & Dean, L. A. (1942). Zinc deficiency in relation to soil and crop composition. *Soil. Sci.* **54**, 315.

MacGillavry, D. & Rideal, E. K. (1937). On the theory of limiting currents. *Rec. trav. chim. Pays-Bas*, **56**, 1013, 1039.

McReady, R. M. & Hassid, W. Z. (1944). Preparation and purification of glucose-l-phosphate by the aid of ion exchange adsorbents. *J. Amer. Chem. Soc.* **66**, 560.

Majer, V. (1937). Changes of polarization when using small anodes. *Collection Czechoslov. Chem. Commun.* **9**, 360, 459.

Manning, W. M. (1940). Continuous records of dissolved oxygen in lake waters. *Ecology*, **21**, 509.

Moore, S., Stein, W. H. & Bergmann, M. (1942). Protein constituent analysis by the solubility method. *Chem. Rev.* **30**, 423.

MÜLLER, O. H. (1939). Oxidation and reduction of organic compounds at the dropping mercury electrode. *Chem. Rev.* **24**, 95.

MÜLLER, O. H. & DAVIS, J. S. (1945). Polarographic studies of proteins and their degradation products. *J. Biol. Chem.* **159**, 667.

MÜLLER, O. H., GARMAN, R. L., DROZ, M. E. & PETRAS, J. (1938). The cathode ray polarograph. *Industr. Engng Chem.* (Anal. ed.), **10**, 339.

MYERS, F. J. (1943). Ion exchange resins. *Industr. Engng Chem.* **35**, 858.

NICHOLS, M. L. & ROGERS, L. H. (1944). Determination of small amounts of molybdenum in plants and soils. *Industr. Engng Chem.* (Anal. ed.), **16**, 137.

NICKELSON, A. S. (1946). Inorganic applications of polarographic analysis. *Analyst*, **71**, 58.

ORLEMANN, E. F. & KOLTHOFF, I. M. (1942). Reduction of iodate and bromate ions. *J. Amer. Chem. Soc.* **64**, 1044, 1676.

PASTERNAK, R. (1947). Komplexbildung von Benzil mit Borsäure. Gleichgewichts- und Reaktionsgeschwindigkeits-Messungen mit den Polarographen. *Helv. chim. Acta.* **30**, 1984.

PASTERNAK, R. & v. HALBEN, (1946). Polarographische Untersuchungen an organischen Verbindungen. *Helv. chim. Acta*, **29**, 190.

PETERING, H. G. & DANIELS, F. J. (1938). Determination of dissolved oxygen with applications to biology. *J. Amer. Chem. Soc.* **60**, 3796.

PIPER, C. S. (1942). *Soil and Plant Analysis.* Univ. of Adelaide.

RANDLES, J. E. B. (1947). Application of cathode ray oscillograph to polarography. *Analyst*, **72**, 301.

RAYMAN, B. (1931). Effect of some organic dyes on maxima of current due to electroreduction of oxygen. *Collection Czechoslov. Chem. Commun.* **3**, 314.

REED, J. F. & CUMMINGS, R. W. (1940). Determination of zinc in plant materials. *Industr. Engng Chem.* (Anal. ed.), **12**, 489.

REED, J. F. & CUMMINGS, R. W. (1941). Determination of copper in plant materials. *Industr. Engng Chem.* (Anal. ed.), **13**, 124.

RICHES, J. P. R. (1946*a*). Supporting electrolytes in polarographic analysis. *Nature, Lond.*, **157**, 479.

RICHES, J. P. R. (1946*b*). A dropping mercury electrode of improved sensitivity. *Nature, Lond.*, **157**, 520.

RICHES, J. P. R. (1946*c*). The use of synthetic resins in the estimation of trace elements. *Nature, Lond.*, **158**, 96.

RICHES, J. P. R. (1947). Preliminary experiments on the use of synthetic resins in the estimation of trace elements. *Chem. Ind.* p. 656.

ROBERTS, E. R. (1941). The polarographic behaviour of histidine and certain other amino acids. *Trans. Faraday Soc.* **37**, 353.

SCOTT, E. W. & BAMBUCH, K. (1942). Polarographic determination of nitrates in blood and urine. *Industr. Engng Chem.* (Anal. ed.), **14**, 136.

SCOTT, R. O. & MITCHELL, R. L. (1943). Concentration methods of spectrographic analysis. *J. Soc. Chem. Ind., Lond.*, **62**, 4.

STERN, A., BEACH, E. F. & MACY, I. G. (1939). Polarographic microdetermination of cystine in protein hydrolysates. *J. Biol. Chem.* **130**, 733, 741.

STILES, W. & SKELDING, A. D. (1940). The salt relations of plant tissues. II. The absorption of manganese salts by storage tissue. *Ann. Bot.* N.S. IV, 673.

STONE, K. G. & FURMAN, V. H. (1944). Polarographic use of organic reagents. *Industr. Engng Chem.* (Anal. ed.), **16**, 596.

WALKER, B. S. (1941). Amperometric measurement of the potency of catalase. *J. Amer. Chem. Soc.* **63**, 2015.

WALKLEY, A. (1942). Determination of zinc in plant materials. *Aust. J. Exp. Biol. Med. Sci.* **20**, 139.

WICHMANN, H. J. (1939). The dithizone system. *Industr. Engng Chem.* (Anal. ed.), **11**, 66.

WIESNER, K. (1947). The overvoltage of oxido-reductions at the dropping mercury electrode, caused by the adsorption layer of dyes of the eosin group. *Collection Czechoslov. Chem. Commun.* **12**, 594.

AN APPARATUS FOR THE EXTRACTION OF GAS FROM SMALL AMOUNTS OF PLANT SAP OR TISSUE

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(With 2 figures in the text)

An apparatus for the extraction of gas from plant tissue may be designed to remove the whole of the gas, dissolved as well as intercellular (Denny, 1946), or to sample only intercellular gas (Kidd, 1937; Wardlaw & Leonard, 1939; Smith, 1947). The apparatus described below is of the former type and was designed for use with sap or tissue of the order of 1 ml. in volume. The principle of the method—extraction of the gas in a vacuum—is simple and has often been used (see, for example, Magness, 1920; Brooks, 1937; Denny, 1946), but the use of small amounts of material necessitated modifications which avoided various sources of error which were not serious when the volume of extracted gas was large. Thus, for example, stopcocks were excluded from positions such that they might cause leaks into the evacuated chamber. A leak of the order of 0·0001 ml./hr. might appreciably affect the results obtained.

Description of apparatus

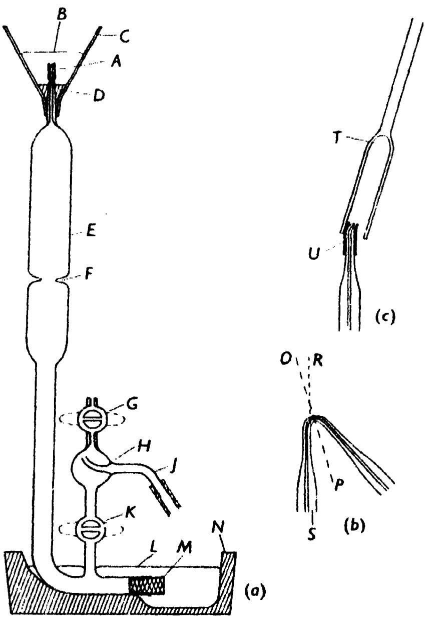

Fig. 1. Extraction apparatus.

The apparatus is illustrated in Fig. 1 (*a*). It consisted of a tube about 20 cm. long and 1·2 cm. in internal diameter, sealed on to an extraction chamber (*E*) about 15 cm. long and 2·5 cm. in diameter. The top of the extraction chamber was closed by a valve (*A*) immersed in mercury contained in a funnel (*C*) sealed on to the stem of the valve by Everett's wax (*D*). The lower end of the tube was bent smoothly at right angles and immersed in mercury (*L*) contained in a trough (*N*) scooped out of a 1 lb. block of B.D.H. paraffin wax. The hollow scooped in the block was just large enough at one end to contain the tube. At the other end it was made wider and deeper to permit the ready manipulation of cores of tissue under the surface of the mercury. The apparatus was connected to a mercury reservoir by means of pressure tubing attached to a side arm (*J*) leading into a trap (*H*) designed to avoid the possibility of air bubbles (which might leak through the pressure tubing) being carried into the apparatus with the mercury. In Fig. 1 the side arm is shown, for clarity, at right angles to its actual position which should be depicted perpendicular to the plane of the paper. The construction of the valve is shown more clearly in Fig. 1 (*b*). A piece of capillary tubing (external diameter 6 mm.; bore 1 mm.) was drawn out to a thickness of about 2·5 mm., bent over sharply at the constricted part, and cut in two (in the line *OP*) at the bend. The part retained for use as a valve was then ground down to the plane *RS*, on a No. 109 'Carborundum' sharpening stone, to give a smooth semi-cylindrical face. The capillary tubing as thus prepared was sealed on to the top of the extraction chamber.

PREPARATION FOR USE

The apparatus was cleaned with chromic acid, well rinsed with tap water and distilled water, and clamped in a vertical position with the bottom end under mercury in the trough (*N*), the stopcocks *G* and *K* being open. The open end was then closed with a rubber bung (*M*) and the mercury reservoir attached to the side arm (*J*). Clean mercury was allowed to run in slowly from the reservoir until it overflowed from the open end of the capillary tubing at the top, stopcock *G* being turned off when the trap *H* was completely full of mercury. Mercury was then poured into the funnel *C* to the level *B* and stopcock *K* closed. A 1 cm. length of bicycle valve tubing was squeezed between the thumb and finger under the mercury in the funnel, and then slipped over the end of the capillary tubing, thus forming a valve similar in principle to a bicycle valve. The extraction chamber, and as much of the tube below it as possible, were rapidly evacuated and filled three or four times by lowering the reservoir with stopcock *K* open, closing the stopcock, raising the reservoir, and opening the stopcock to allow mercury to flow into the apparatus as quickly as possible until it squirted from the valve *A*. The extraction chamber was finally evacuated and left in this condition for at least as long as the time which the extraction of gas from the tissue or liquid was expected to take. During this period the stopcock *K* was closed and the reservoir placed in the 'raised' position to lessen the danger of air leakage through the pressure tubing. In practice we found it convenient to prepare the apparatus in the evening and leave it evacuated overnight ready for use the next day.

METHOD OF GAS EXTRACTION

A. *From borings of potato tissue*

The reservoir was lowered and the stopcock *K* opened, thus transferring to the trap *H* any air which had leaked into the stopcock. The latter was then partially closed and the reservoir raised, the apparatus being thus slowly filled with mercury. The stopcock was then closed, and the bung *M* removed.

A cork-borer (internal diameter 1 cm.), filled with boiled water, and with no air bubbles adhering inside, was plunged through the tuber, held under boiled water and with no air bubbles adhering. The boring was expelled under mercury in the trough *N*, its ends were rejected, and a section about 1–1·5 cm. long was inserted into the open end of the apparatus and propelled up the tube by a length of steel wire, being prevented from going right to the top by the indentations *F*. The bung was then replaced, the stopcock *K* opened, and the mercury reservoir lowered until the extraction chamber was evacuated, after which the stopcock was closed and the reservoir raised. The time taken in our experiments between first plunging the cork-borer into the tuber and evacuating the extraction chamber was 45–60 sec.

From the results given in Table 1 it will be seen that about 95% of, for example, the nitrogen was extracted in half an hour, and extraction was practically complete after 5·5 hr. The gas extracted was ejected from the apparatus by slightly opening the stopcock

K, and collected in a mercury-filled tube (Fig. 1 (*c*), *T*) held over the valve at an angle such that the valve tubing (*U*) was pushed away from the orifice of the valve (see Fig. 1 (*c*)). It was then transferred over mercury to a Bonnier-Mangin gas analysis apparatus.

Table 1. *Nitrogen extracted from potato tuber borings, held in vacuum for varying periods of time, expressed as a percentage of the volume extracted after 23·5 hr.**

Time in vacuum (hr.)	Percentage of volume		
	Boring no. 1	Boring no. 2	Boring no. 3
0·5	94·8	94·7	95·3
1·5	96·5	95·7	96·3
3·5	97·9	97·1	98·1
5·5	99·5	99·6	99·6
23·5	100·0	100·0	100·0

* Extraction for longer than 23·5 hr. produced no measurable increase in the volume.

B. *From liquids*

Before filling the apparatus with mercury and clearing the air from it, as under 'Preparation for use' above, two 4 × 1 cm. pieces of a 'Whatman' ashless filter tablet were inserted into the open end of the tube and pushed into the extraction chamber with a length of steel wire. Apart from this the apparatus was prepared as previously described and before use the stopcock *K* was cleared of air and the apparatus filled with mercury as under 'A' above. The stopcock was then closed, the valve tubing removed from the valve, and the mercury reservoir lowered to an intermediate position at about the level of the stopcock *G*.

The liquid from which the gas was to be extracted was placed in a 1 ml. burette to the delivery end of which, held under the surface of part of the residue of the liquid, was attached a 1 cm. length of valve tubing. The tap of the burette was opened until a drop formed at the end of the valve tubing, then closed, and the free end of the tubing slipped over the valve of the extraction apparatus, slightly off centre to leave clear a channel from the burette to the valve orifice. The burette tap was opened and the desired amount of liquid drawn into the apparatus by manipulation of stopcock *K*. Burette and tubing were then disconnected with the burette tap still open. The stopcock *K* was opened sufficiently to draw a little mercury into the extraction chamber from the funnel *C*, thus clearing the liquid from the upper part of the valve, over which the original length of valve tubing was finally slipped, care being taken to expel all the air from it first. The mercury reservoir was then completely lowered and the stopcock *K* opened until the extraction chamber was emptied of mercury, when the stopcock was closed and the reservoir raised. The gas extracted was ejected as under 'A' above, but the liquid was retained by the filter tablets in which it had been absorbed when it was drawn back into the extraction chamber. Re-solution of the extracted gas and the introduction of liquid into the Bonnier-Mangin apparatus were thus avoided. We found an extraction period of 2 hr. to be adequate for the extraction of oxygen and nitrogen from the liquids we used (distilled water and potato sap).

METHOD OF GAS ANALYSIS

The gas extracted could be analysed with sufficient accuracy for our purpose in a Bonnier-Mangin apparatus (Aubert, 1891; Thoday, 1913). We used a tube with a bore of the order of 1 mm. (1 cm. in length was equivalent to a volume of 0·00827 ml.) and took readings with the aid of a watchmaker's lens. The oxygen-absorbent employed was a 10% alkaline solution of 1–2–4 triacetoxybenzene. The results of analyses of small volumes of air obtained during routine calibration of the apparatus are given in Table 2. With such small volumes of gas the accuracy of the analysis is of course not so great as with larger volumes (see Thoday, 1913).

Table 2. *Analyses of small volumes of* CO_2*-free air in the Bonnier-Mangin apparatus*

Vol. of sample (ml.)	% O_2	% N_2 (by diff.)	Vol. of sample (ml.)	% O_2	% N_2 (by diff.)
0·075	20·8	79·2	0·028	20·9	79·1
0·043	20·9	79·1	0·025	20·8	79·2
0·039	20·4	79·6	0·025	20·4	79·6
0·037	20·7	79·3	0·020	20·8	79·2
0·031	20·6	79·4	0·017	20·7	79·3
0·029	20·4	79·6	0·016	21·2	78·8

ACCURACY OF THE METHOD

The degree of accuracy obtained can be seen from Fig. 2, in which the circles represent the volumes of gas, corrected to N.T.P., extracted from 1 ml. of distilled water saturated with air at various temperatures. The continuous lines represent the values given in Landolt-Börnstein (1923), in the *International Critical Tables* (1928) and in the *Handbook of Chemistry and Physics* (1945). They are largely based on the results of Winkler, who used about 2 l. of water.

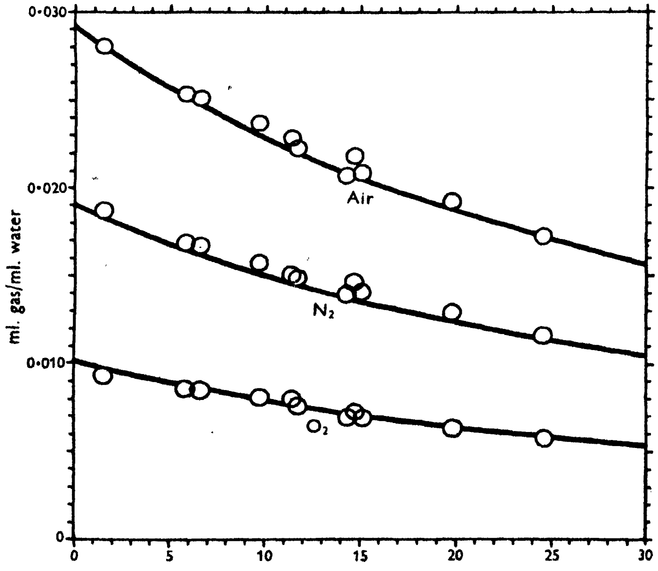

Fig. 2. Volumes of gas, at N.T.P., extracted from 1 ml. of distilled water saturated with air at various temperatures.

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REFERENCES

AUBERT, M. E. (1891). Nouvel appareil de Mm. G. Bonnier et L. Mangin pour l'analyse des gaz. *Rev. gén. Bot.* **3**, 97.

BROOKS, C. (1937). An apparatus for the extraction of internal atmospheres from fruits and vegetables. *Proc. Amer. Soc. hort. Sci.* **35**, 202.

DENNY, F. E. (1946). Gas content of plant tissue and respiration measurements. *Contr. Boyce Thompson Inst.* **14**, 257.

Handbook of Chemistry and Physics (1945). Ed. C. D. Hodgman. Cleveland, Ohio: Chemical Rubber Publishing Co. 29th edn. 2640 pp.

International Critical Tables of Numerical Data, Physics, Chemistry and Technology (1928). Ed. E. W. Washburn *et al.* Vol. III, 444 pp. New York: McGraw-Hill.

KIDD, F. (1937). The internal atmosphere of potatoes. *Rep. Food Invest. Bd., Lond.*, 1937, p. 179.

LANDOLT-BÖRNSTEIN (1923). *Physikalisch-Chemische Tabellen.* 5th edn., Vol. I, 784 pp. Berlin: Springer.

MAGNESS, J. R. (1920). Composition of gases in intercellular spaces of apples and potatoes. *Bot. Gaz.* **70**, 308.

SMITH, W. H. (1947). A new method for the determination of the composition of the internal atmosphere of fleshy plant organs. *Ann Bot., Lond.* (N.S.), **11**, 363.

THODAY, D. (1913). On the capillary eudiometric apparatus of Bonnier and Mangin for the analysis of air in investigating the gaseous exchanges of plants. *Ann. Bot., Lond.*, **27**, 565.

WARDLAW, C. W. & LEONARD, E. R. (1939). Studies in tropical fruits. IV. Methods in the investigation of respiration with special reference to the banana. *Ann. Bot., Lond.* (N.S.), **3**, 27.

STUDIES IN THE BASIDIUM

I. THE AMPOULE EFFECT, WITH A NOTE ON NOMENCLATURE

By E. J. H. CORNER, M.A., F.L.S.

(With 9 figures in the text)

CONTENTS

PAGE
Introduction 22
  The basidium unit in *Oudemansiella Canarii* 23
  Seven stages in development 25
  The mechanism of the basidium 31
  Proof of the hypothesis 32
  Spore-basidium-volume 33
  Spore size and spore number 41
  Three exceptions 43
  The ampoule effect 48
  The nomenclature of the basidium unit 49
Summary 51
References 51

INTRODUCTION

That the basidium empties its contents into the spores and, in doing so, becomes filled with a vacuole, has been well known since de Bary's description of the process in *Aleurodiscus* (*Corticium*) *amorphus* (1884, p. 68, f. 30). But it was Buller who suggested, in 1933, that the transference was effected by the same method of vacuolar pressure as caused protoplasmic streaming in hyphae. He observed that protoplasm was often pushed from cell to cell through the septal pores by enlargement of a vacuole, or group of vacuoles: thus, in the mycelium of *Pyronema confluens*, the protoplasm was seen to travel through as many as 161 cells, or over 16 mm. length of a hypha. He concluded, also, that vacuolar pressure was the motive force of apical growth, the dense growing protoplasm at the hyphal tip being continuously thrust forward by the enlarging vacuoles in the maturing part of the apical cell. He did not observe protoplasmic streaming in basidia but, from the vacuoles which formed at the base of basidia which were developing spores, inferred that it must take place (cf. Buller, 1933, vol. 5, pp. 147–8, for *Coprinus sterquilinus*).

In the last few years I have studied the basidia of several tropical fungi, and am able to give a more comprehensive account of the gross working of the basidium. Without this preliminary picture one cannot proceed to analyse the intimate and complicated relations in size and shape between the basidium and the spores which it produces. It shows that the ***basidium unit***, consisting of the parent cell and its spores, provides material for exact quantitative investigation.

The fungus which I have used mainly is the agaric *Oudemansiella Canarii* (= *Collybia apalosarca*). It is closely related with *Collybia radicata* and *Armillaria mucida*, both of which are now placed in *Oudemansiella*. They are common and typical Basidiomycetes, with large, almost globose, spores, and large basidia which develop rapidly and are easy to observe on account of the very numerous small oil drops in the cytoplasm. (For a general description of *O. Canarii* see Corner, 1934; for the synonymy of *O. Canarii*, see Singer, 1945.)

THE BASIDIUM UNIT IN *OUDEMANSIELLA CANARII*

A free-hand section of a gill from a newly expanded pileus will show, when mounted in water, basidia in all stages of development.

The first stage of Fig. 1 shows a basidium arising from a subhymenial cell. Its cytoplasm is slightly opalescent, like that of a hyphal tip, and has no sign of the abundant reserve material which later appears as oil droplets. The young basidium stretches into the hymenium between the stalks of older basidia, mainly by the enlargement of one to three vacuoles which arise in it at a very early stage (stages 1–3, Fig. 1). When it is about half its final size, the cytoplasm begins to increase at the apex and to form oil droplets (stage 4). The basidium now enlarges mainly by the basipetal accumulation of dense oleaginous cytoplasm. At the same time the vacuole dwindles away, or subdivides into minute vacuoles dispersed throughout the cytoplasm, until the basidium appears to be filled only with dense cytoplasm (stages 5 and 6). A new set of vacuoles then arises in the stalk and by their enlargement, rather than by the accumulation of more protoplasm, the basidium grows to its full size (stage 7). A characteristic feature now appears at the apex. A *hyaline cap* of slightly opalescent, homogeneous cytoplasm develops by displacing the oleaginous cytoplasm (stage 8). Very soon, in a few minutes perhaps, the four sterigmata arise as broad, blunt outgrowths which curve inward and taper to acute, subparallel tips (stage 9): they are filled with this homogeneous cytoplasm. The tips then expand into minute, transversely placed, rods or disks on which the spores develop *abaxially* (away from the long axis of the basidium), while the adaxial projection becomes the apiculus of the spore (stage 10); the spore rudiments, too, are filled with the remaining homogeneous cytoplasm. The next stages (11–13) are simply the outcome of the gradual enlargement of the basal vacuole, the hemispherical top of which pushes the dense protoplasm into the sterigmata and, through their necks, injects a fine stream into each of the spores. One can watch, under a low power of the microscope, the upwards, piston-like, passage of the vacuole from a stage between 10 and 11 (cf. Fig. 1) until the complete vacuolation of stage 13: it takes about half an hour, and it is a continuous slow movement, sweeping up the stray droplets on the sides of the basidium until the apex of the vacuole touches the top of the basidium and moulds itself into the sterigmata. During the period of maximum growth of the spore, between stages 11 and 12, the oil droplets pass into the spore at intervals of 4–15 sec.: they elongate as they approach the sterigmatic neck, and, in passing through it, are drawn out into a dark line which instantly contracts in the apiculus into a refringent guttula and passes into the centre of the spore. Thus, the actual canal in the sterigmatic neck is much finer than the neck itself, which has, as one would expect from mechanical reasons, a slightly thickened wall: judging from the size of the guttula, the canal is about 0·2 μ wide (it should be possible to see the passage of the nucleus from the interruption in the stream of oil droplets, but I failed to note this).

The wall of the spore begins to thicken when it is nearly full grown. The mature spore has a hyaline smooth wall and dense, non-vacuolate, cytoplasm consisting mostly of

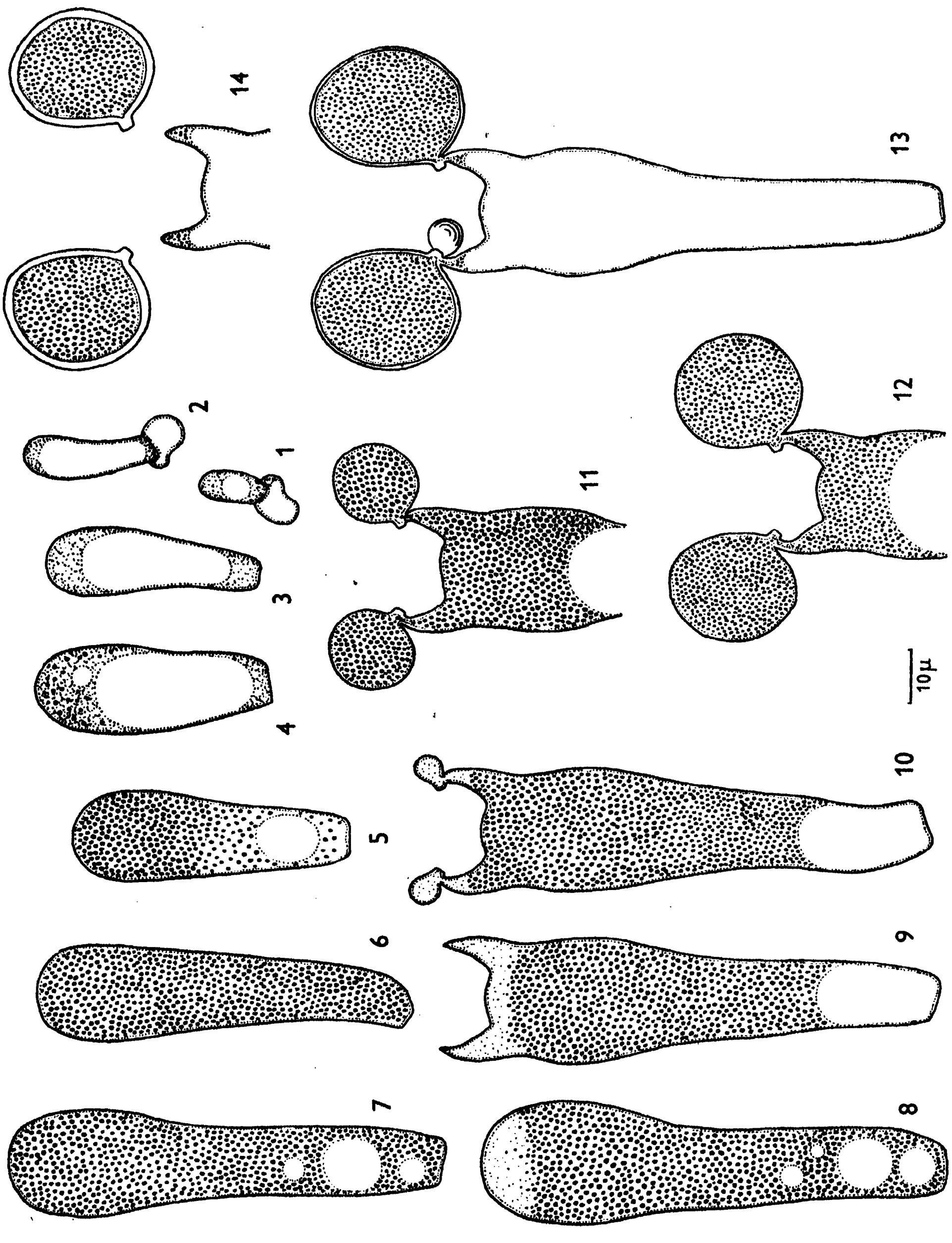

Fig. 1. Stages in the development of the basidium of *Oudemansiella Canarii*, the oil droplets in the cytoplasm shown diagrammatically as black dots; only two opposite spores of the tetrad are shown. × *c.* 800. Stages 1–3, the inception of the basidium; 4, 5 the beginning of charging; 6, the fully-charged, immature basidium; 7, the beginning of the initial vacuolation of the charged basidium; 8, the full-sized basidium with hyaline cap; 9–13, the development of sterigmata and spores; 14, discharge of the spores.

almost contiguous oil droplets (much more crowded than shown in Fig. 1). At maturity, Buller's drop is exuded from the sterigmatic tip on its *adaxial* side (toward the long axis of the basidium), and the spore is shot away to a horizontal distance of 150–250μ. The first two spores are discharged almost or quite simultaneously, the next two successively and, often, with a very small drop, about a quarter the diameter of that on the first two spores. (If a piece of pileus, with the gills cut tangentially so as to be 2 or 3 mm. thick, is set on a small drop of water in a watch-glass with the gills vertical, one can observe the development of the projecting basidia with a low-power objective and high-power ocular: there is an incessant flicking off of spores like a display of golf balls.)

From this general sequence there may be a few variations. Two or three vacuoles may form in stages 2–4 in place of one. Stage 6 may pass directly into stage 8 when the basidium is short, or, in contrast, when the basidium is long it may be wholly vacuolate in the lower third. Finally, the basidium may not become wholly vacuolate: often more or less of the sterigma is full of dense cytoplasm and there may be a thin cap of residual cytoplasm over the apex of the vacuole.

SEVEN STAGES IN DEVELOPMENT

This description of the basidium of *Oudemansiella Canarii* applies in the main to all Homobasidiomycetes, as *Clavaria*, *Craterellus*, *Polyporus*, *Russula*, *Hygrophorus* and so on. It is necessary, or, at least, convenient, to distinguish seven stages which, with reference to Fig. 1, are as follows:

Stage of development	Reference to Fig. 1	Time interval for *Oudemansiella Canarii*
1. Inception	1–3	?
2. Charging	4–6	?
3. Initial vacuolation	7–8	*c.* 2 hr.
4. Development of sterigmata	8–9	*c.* 30 min.
5. Development of spores	10–13	*c.* 45 min. + *c.* 30 min.
6. Discharge	14	1–2 min.
7. Collapse	—	—

The full time of development is probably about 6 hr.*

Inception. This is the preliminary hyphal stage of the basidium (see Corner, 1947). The enlarging vacuole pushes the young basidium into a free space between the stalks of pre-existing basidia and so the basidial stalk is often curved. Because of the large vacuole and clear cytoplasm, this stage may be mistaken for a paraphysis (which seems, in fact, to be a basidium that is never charged).

Charging. This is the stage when the basidium fills up with the dense cytoplasm which is passed into the spores. It is a very striking feature of the microscopic structure of Basidiomycete fruit bodies that only in the developing basidia does the condensation of storage material become visible. Thus, in *Oudemansiella Canarii* and many other fungi with guttulate spores, as *Amanita*, *Russula*, species of *Clavaria*, and so on, the longitudinal hyphae of the general tissue and the interwoven hyphae of the subhymenium have watery transparent contents, in strong contrast with the viscous, guttulate cytoplasm of the basidia. The cytoplasm of the mature spores resembles that of the basidium, though usually the oil droplets in them fuse into one or a few large oil drops. The condensation of anabolites occurs in the developing basidium and not in the developing spores.

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* Details are:
Stages 4–6: 115, 119, 124 min. for three basidia.
Stages 7–8: 25, 28, 31 min. for three basidia.
Stages 10–13: 44, 45, 45, 48, 49·5, 50 min. for six basidia.
Stages 13–14: 25, 25, 27·5, 30·5, 31, 32 min. for six basidia, as a quiescent period of wall-thickening before discharge.

In some fungi, as *Oudemansiella Canarii*, the basidium becomes fully charged with dense (that is, non-vacuolate) cytoplasm. In others, especially with elongate hypha-like basidia, e.g. *Clavaria*, charging occupies only the distal clavate part, the stalk remaining vacuolate.

Initial vacuolation of the full-sized basidium. As will appear later, this conception is needed to relate the size of the basidium with that of its spores. It means the amount of vacuolation of the full-sized basidium. In nearly every case that I have studied, the full-sized basidium, without sterigmata, has a more or less vacuolate stalk. It contains, that is, dense cytoplasm which will be passed into the spores and hydrated or vacuolate cytoplasm which will not. The hydrated part may be newly developed after complete charging (as in *Oudemansiella Canarii*) or it may comprise, also, vacuoles of the stage of inception which have not been occluded during charging (as in *Clavaria*). If, after charging, the wall of the basidium is fully stretched, there can be no vacuolation until the sterigmata emerge, but this seems rarely the case. The new vacuolation, which begins after charging and which finally distends the basidium in *Oudemansiella Canarii*, starts as a few minute vacuoles in the proximal half, but they soon unite into the large basal vacuole. Up to this stage in development, the basidium has been more or less immersed in the palisade of the hymenium: indeed, the surface level of the hymenium-palisade is, practically, the level reached by the tops of the fully charged basidia. During the vacuolation which follows charging, the top of the basidium projects so as to get the free room for the development of the spores. According to the amount of this vacuolation, so the full-sized basidia project more or less and, if the degree of projection is organized, the result is seen in the complex agaric-hymenia with di-, tri- and tetramorphic basidia, giving two, three or four spore levels (as described by Buller). Thus, the full-sized basidium with projecting top is under high tension: its distended apex compresses the dense cytoplasm against the basal vacuole (or vacuoles) which bear against the septum, and this intrinsic tension is heightened by lateral compression of the basidium in the hymenium caused by the continued insertion of new basidia round its base. The tension is relieved by the development of the sterigmata.

Development of the sterigmata. Not until the basidium is full sized do the sterigmata appear. If the basidium enlarged after their appearance, the symmetry of the spores would be upset and, probably, monstrous spores of various sizes and shapes would result. Full-grown rigidity is necessary for the perfection of the basidium unit and this is indicated, so far as I have found in a wide range of Homobasidiomycetes, by the appearance of the hyaline cap. It indicates that the basidium is full sized and about to form the sterigmata. When no hyaline cap can be seen in the living basidium (as in some *Clavarias*), the effect is caused, I think, by lack of optical distinction between it and the dense cytoplasm in the basidium, rather than by any difference in procedure. In relatively broad basidia (e.g. *Boletus*, *Amauroderma*), the hyaline cap may be so thin as to be barely distinguishable from the wall of the basidium: possibly, even, in *Amauroderma*, it is in four discrete patches where the widely spaced sterigmata arise.

Now, the hyaline cap represents a rearrangement of the cytoplasmic constituents at the apex of the basidium. It is obvious in multiguttulate basidia, for the guttulae are cleared away from the apex, but it is not so evident in basidia with merely opalescent contents, as those of *Omphalia* or *Pistillaria*. The hyaline cap resembles, in fact, the tip of a hypha and, just as the hyphal tip must primarily make wall material, so it seems that the hyaline cap is the wall material assembled in position for the sterigmata and young spores. On extrusion it is shaped into the sterigmata and the spore rudiments, which have the same vitreous transparency, and through the middle of these two bodies, assembled as a pair of asymmetric cones, the dense cytoplasm of the basidium passes in a fine canal.*

The sterigmata develop more or less simultaneously as blunt protuberances where the sides of the basidium arch over to the top.† They must be preceded by local softenings of the basidium wall which are then forced out by the internal pressure of the basidium.

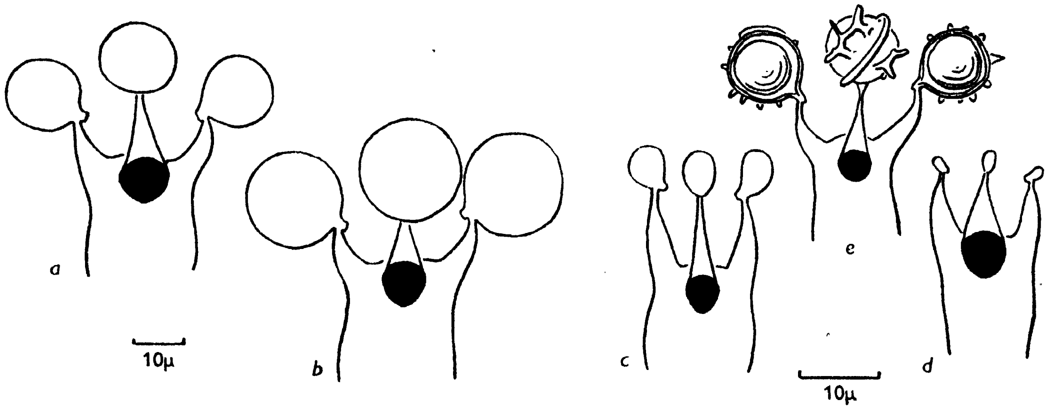

Fig. 2. Distal ends of basidia to show the shape of the sterigmatic base (in black); only three spores of the tetrad are shown. *a*, *b*, *Oudemansiella Canarii*, with immature and mature spores, × *c*. 500; *c*, *d*, *Clavaria pistillaris*, with immature spores, × *c*. 900; *e*, *Russula* sp. (aff. *R. froetens*), × *c*. 900.

One must postulate a ***sterigmatic patch*** on the basidium for each sterigma as a special subapical area of the wall which develops after the hyaline cap and which initiates the working of the basidium by providing an outlet. To judge from the shape of the sterigmatic base, as shown in Fig. 2, the sterigmatic patch must be ovate, with the blunt end toward the basidium apex. The sterigmata are, at first, blunt protuberances (not points) which are normal to the surface, but they soon curve adaxially until they are ***subparallel*** and then they taper to acute tips. The sterigmata harden and narrow against the increasing pressure in the basidium. The adaxial curvature must be caused by the more rapid hardening, or fixation, of the sterigmatic wall on its adaxial side. Thus, the adaxial side is shorter and the hardening, proceeding acropetally, reaches the sterigmatic tip sooner on its ***adaxial*** side than on its abaxial, for which reason, perhaps, the next stage is introduced by the ***abaxial*** outgrowth of the spore rudiment.

Development of the spores. The tip of the contracted sterigma expands into a minute disk, placed at right angles to the long axis of the basidium, and this disk swells abaxially into the spore. At first, the remainder of the hyaline cap passes into the spores; then, the dense cytoplasm of the basidium flows in and swells them to their full size. The slight adaxial projection of the primordial disk forms the apiculus of the spore. The shape of the spore depends on the manner in which its wall thickens and hardens during development. If the wall remains thin and equally distensible in all parts, a globose (or subglobose) spore is produced. Proof of the distensibility of the wall was given by an abnormal basidium of *Oudemansiella Canarii* in which I saw the oil droplets streaming down one sterigma as its immature spore, 10 μ wide, began to shrink: at the same time, an adjacent spore began to swell rapidly until in 5 or 6 sec., when it had nearly doubled its diameter, it burst, and the whole basidium collapsed: the shrinking spore had dwindled to a sphere 4 μ wide and the enlarging one had also remained globose. On reaching its full size, the wall of the globose spore thickens all over, but more particularly at the apiculus which appears solid.

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* Thus unlike the extravagination of invaginated parts of animal cells, as the nematocysts of Coelenterata.

† It is sometimes stated that the centrosomes of the daughter-nuclei of the basidium move to its apex and that, where they touch the wall, the sterigmata arise. Later studies have shown that this conclusion is based on an erroneous interpretation of the sequence of events in the basidium and that both sterigmata and young spores arise independently of the nuclei, as do hyphal branches: indeed spores may be anuclear (Smith, 1934, p. 305).

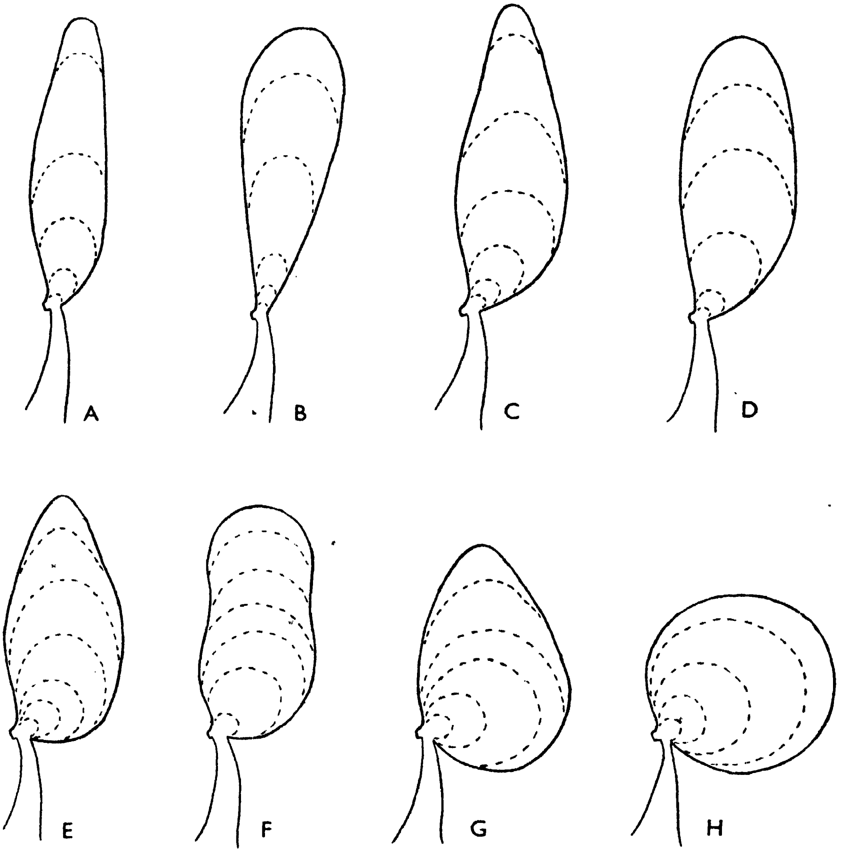

Fig. 3. Diagrams of developing basidiospores. A–D, with the wall rapidly hardening acropetally, A and C, with attenuate tips; E–H, with the progressively delayed acropetal hardening and progressively increasing abaxial bulge, leading to the globose spore H; E, the amygdaliform, or mango-shaped, spore; F, the waisted spore; G, the spore with 'lateral apiculus'.

In contrast, in ellipsoid or elongate spores, the wall begins to harden and thicken acropetally from the apiculus while the spore is still enlarging. Thus, according to the relative rates of acropetal fixation of the wall and of the enlargement of the spore, so differently shaped elongate spores result, as shown in Fig. 3. Thus, at the opposite extreme to the globose spore, are the elongate pip-shaped spore and the rod-like spore in which acropetal fixation begins at an early stage and the apex of the spore grows as a hyphal tip. Eventually the wall thickens, more or less, and, in elongate spores with distinctly thickened walls, it can usually be seen that the thickening, like the fixation, has been acropetal, as shown in Fig. 4. In such, also, the apiculus appears to be solid at maturity, and that the base of the spore has the thicker wall is indicated by the fact that thick-walled basidiospores usually germinate from apex.

In developing, the spore bulges little, if at all, adaxially, and thus its adaxial side appears flattened compared with the convex abaxial side. The extreme case is the arcuate spore, as in species of *Merulius*, which is concave on its adaxial side, very much as the sterigma.

Discharge of the basidium. This process has been amply described by Buller, and I can add little except to confirm the explanation of spore discharge which he tentatively suggested (Buller, 1922, vol. 2, pp. 24–5). The drop, which appears at the apiculus just before the spore is shot off, is, I think, not a factor in the mechanism but an incident, as Buller suggested, which shows when the act is about to begin.

The mature basidium is fully turgid and its stalk is squeezed by the pressure of young basidia developing around it. It has the rigidity to project horizontally and to bear the large spores at its free end on slender spikes: the bending moment of the spore about the tip and base of the sterigma must be considerable.*

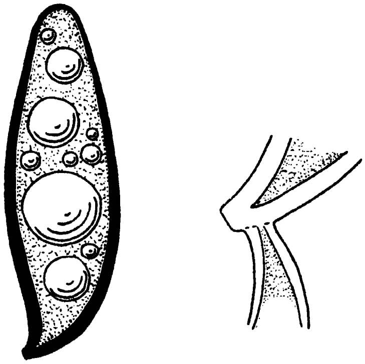

Fig. 4. A diagram of a basidiospore to show acropetal thickening of the wall, and a diagram of the possible apicular structure at maturity of the spore.

Just before the spore is shot off, the tip of the sterigma must weaken, by dissolution or by becoming mucilaginous. A moment will come when the cohesion of the wall in this zone equals the internal pressure of the basidium. A moment later, the wall breaks and, presumably, a minute jet of cytoplasm impinges on the apiculus and shoots off the spore. Buller's drop, which exudes a few seconds earlier, indicates the weakening of the wall, because there is evidence, as I will now show, that it is merely cytoplasm or vacuolar sap forced through the wall.

Buller discussed the chemical nature of the drop as if it were an aqueous solution. Such, however, would certainly flow over the spore and down the sterigma. Indeed, Buller describes how the drop, on being carried away by the shot-off spore, flows over it. Moreover, microscopic drops of water, condensed on a dry coverslip resting on a gill, flow round the spore tetrads and on to the basidia, the apices of which are more or less rapidly damaged by wetting.* The problem is why the exudate should be a drop while the spore is still attached and then flow over it when carried off on the detached spore.

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* Cf. Ingold (1939, f. 36). The basidia of some species of *Boletus* and *Marasmius* with elongate spores 20–30 × 3–7 μ, widest at the middle or near the apex, are very striking. The basidium can be likened to a rubber glove with each finger tip expanded into a hollow ball or sausage, then filled with water, held horizontally and squeezed from the wrist until it is rigid.

I have seen the drop in its normal position in fruit bodies of *Clavaria pistillaris* which I had preserved in alcohol formalin for 13 years: it appeared as a spherical oleaginous blob of the same appearance as the sterigmatic contents. I was watching, also, spore development in a thick slice of the fruit body of the tropical *Stereum aterrimum* (with 2-spored basidia). One spore of a pair had developed a vitreous drop, 4μ wide, when it began to recede: in some 10 sec. it had been reabsorbed into the sterigma and, in a few minutes, the basidium collapsed.

These observations suffice to show that the drop is surrounded by a membrane, and not until the membrane is ruptured by the shooting off of the spore is the drop normally free to flow. The membrane must be an outer layer of the wall of the sterigmatic neck and the liquid inside must be residual dense cytoplasm with, possibly, some vacuolar sap from the basidium. Such a membrane often, if not always, covers the exudates from cystidia which are in a similar state of turgor and, presumably, excessive permeability of the wall at the tapered apex.

I conclude, therefore, that the discharge of the basidium is caused by hydrostatic pressure, as in the case of the ascus, but that each basidiospore has its own minute jet which, however brief, impinging on the apiculus, causes the spore to revolve in its passage through the air, and the apiculus, moving first, sweeps off the exuded drop which spreads over the spore. The adhesiveness of freshly fallen basidiospores must be caused by the drying film of cytoplasm carried away on the spore. I see no reason to invoke an unknown force, as Buller's alternative, and it is difficult to evaluate the effect of surface tension, as calculated by Ingold (1939, pp. 81–6, f. 38), for the material of the drop seems to be very different from that assumed by him and Buller. The real problems in the discharge of basidiospores seem to be:

(i) What causes the dissolution, or weakening, of the wall of the sterigmatic neck? (This problem is not unique but related with the increased permeability of the wall round the pseudo-sterigmatic tips of cystidia.)

(ii) What causes the almost instantaneous closure of the ruptured sterigmatic neck?

(iii) What causes the adaxial position of the drop?

In connexion with the first point, it should be noted that there is always a considerable interval of time between the attainment of full size by the spore on the sterigma and the appearance of the drop which immediately precedes its discharge. The time needed to dissolve the rigid wall of the sterigmatic neck may account for the interval.

In connexion with the second point, it seems from experiments in micro-manipulation that living protoplasm can instantly repair a minute rupture or perforation in its wall, and thus seal its egress. For the third, it seems that the incurvature of the sterigma with its ovate cross-section directs the maximum flow of cytoplasm adaxially and thus causes the exudation where the apiculus joins the sterigma.

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* If living basidia are mounted in water, they do not swell or burst but sooner or later their contents become granular and disintegrate. Long before this happens, the sterigmata at any stage of spore development bend, and even jump, inward, through an angle of roughly 45°, as though the small apical dome of the basidium, which struts them, had collapsed. The spores, then, are more or less clumped together and fail to develop properly. The basidia of *Clitopilus* and *Ganoderma* suffer after 1 or 2 min. in water. Whatever the physical cause, there can be no doubt that the tops of basidia, which are normally projected into the air for developing spores, are easily wetted and damaged. Clumped tetrads of mature and immature spores are often to be found on the hymenia of *Clavarias* which are exposed to rain, and I think that this fact must have contributed greatly to the survival value of pileate fruit bodies with the hymenium protected from wetting.

It might be expected that the discharge of the basidium would be accompanied by some change in shape, size or position of the sterigmata or basidium, as happens with the ascus on recoil. Such has never been seen either by Buller, Ingold or myself. The sterigmatic jets must be so minute, as they are momentary, that their release will not affect the size or shape of the turgid basidium, which can always absorb more water from its subhymenial cell. The sterigmata have rigid walls and could move only by deformation of the basidium apex.

Collapse of the basidium. After discharge, the basidium slowly collapses by loss of turgor and by compression between developing basidia. Its lining layer of cytoplasm separates from the wall and often becomes a yellowish brown or, even, purplish strand along the centre of the basidium, and thus contributes to the colour of the hymenium. The sterigmata retain their shape for a long time, even indefinitely in species with thickening hymenium (*Clavaria*, *Stereum* and so on), and if in such there is little compression of the old basidia, they too remain clavate and may be mistaken for immersed cystidia. These details show that the basidium wall has its own rigidity apart from that caused by turgor pressure.

Conclusion. Of the seven stages in the life of the basidium, three stand out conspicuously. They are the *fully charged basidium* (Fig. 1, stage 6), the *full-sized basidium* (Fig. 1, stage 8) and the *mature basidium* (Fig. 1, stage 13). In most species, the stages follow in continuous sequence. In some, however, there appear to be pauses, but I am not certain of their exact nature. A pause may occur between inception and charging or, as seems more usual, between charging and attainment of full size by protrusion of the tip: between protrusion and discharge there is never a pause. Thus, in species of *Marasmius*, *Physalacria* and *Pterula* and in numerous 'Corticia', the hymenium in the daytime consists of young and charged basidia and it seems that they do not emerge until night. In developing agaric fruit bodies, the basidia may persist for several days in the state of inception, with new ones proliferating from the subhymenium to close the gaps which would form in the enlarging gills until, on expansion of the fruit body, they charge and begin to work. In Buller's inaequihymeniferous type of agaric, a pause must occur in some stages so that the basidia mature rhythmically in limited areas of the hymenium. In the Heterobasidiomycetes the pauses have been variously elaborated, as in the Uredineae.

THE MECHANISM OF THE BASIDIUM

The basidium is seen to be a terminal cell which condenses protoplasm and passes it into exogenous spores which it then shoots into the air. Observation suggests that the mechanism is simply vacuolar pressure from the base of the basidium acting on various modifications of the wall at its apex. Thus, the expanding vacuole blows out the spores as bubbles of protoplasm, but they are pedicelled in a particular way suited to their abstriction, that is by an exceedingly narrow neck which finally ruptures. The motive force of vacuolar pressure is a common hyphal property, as Buller has shown, but the apical modifications of the wall are the distinction of the basidium. *A priori*, one might have supposed that the sterigmata developed of their own accord as rigid pseudopodia or localized germ tubes from the basidium apex, and that the spores grew as discrete cells which drew their food supply from the basidium as it does from the subhymenial hyphae: that is, the spores might be the condensers, not the basidium. That such suppositions are incorrect is shown by the observations:

(i) that the emergence of the sterigmata coincides with the appearance and enlargement of vacuoles at the base of the full-sized basidium;

(ii) that the material of the hyaline cap first enters the sterigmata and the spore rudiments, exactly as would be expected if they were simply forced out by pressure from below;

(iii) that one can then see the dense cytoplasm streaming slowly into the spores, and, *pari passu*, the vacuole of the basidium advancing towards its apex;

(iv) that when the mature spore is shut off from the sterigma, the drop of residual cytoplasm is forced out of the sterigmatic neck (and, if the process is abnormally prolonged, vacuolar sap of the basidium may also exude until the basidium collapses ineffectively; cf. Buller's abnormally large drops);

(v) that no vacuoles form in the developing sterigmata and spores.

The remarkable and important point is that the maturing of the spores coincides more or less exactly with the emptying of the basidium: the spores become thick-walled and shut off from the sterigmata just as the vacuole approaches their necks. Thus, as one would expect for a resting stage, the spore fills only with dense cytoplasm and such condensed storage material as oil droplets: indeed, in the passing of vacuoles to the base of the basidium at the end of charging (Fig. 1, stages 5–6), one can see the protoplasm ridding itself, as it were, of all excess water and readily hydrated constituents. Nevertheless, after discharge, the spores dry yet further, shrinking and often flattening, and only when they germinate do their contents vacuolate for the extrusion of the germ tube.*

In addition to vacuolar pressure and modification of the basidium wall, one must add a co-ordinating factor which prevents the spore from being filled with vacuole or from becoming excessively enlarged. But how the factor works, whether by fixation of the spore wall or by occlusion of the apicular canal as an outcome of acropetal thickening of the spore wall, seems impossible to decide by observation alone, because of the minuteness of the structure. If, by micro-manipulation, spores can be lanced or nipped off sterigmata, decisive evidence may be obtained.

PROOF OF THE HYPOTHESIS

If the basidiospores develop by the injection of cytoplasm into them from the basidium until it is practically emptied, it follows that:

(i) the spore contents must resemble the basidium contents;

(ii) the size of the spore will depend on the amount of dense protoplasm in the basidium and the number of spores borne by it.

Concerning the first point, there is ample evidence. Thus, multiguttulate spores develop from multiguttulate basidia, as in *Oudemansiella Canarii*, and opalescent spores from opalescent basidia, as in *Omphalia*, *Hygrophorus* and so on. Often the oil droplets fuse into one to three large drops in the spore, but this is no fundamental distinction. In *Clavaria helicoides*, the pink cytoplasm of the basidium passes into the spores and causes them to give a pink spore print. In *Boletus*, *Ramaria* and most 'ochrosporous' genera, there is often a brownish tinge to the cytoplasm of the basidia and this appears to be the pigment deposited in the walls of their spores. There is, in fact, no evidence that the cytoplasm of the spore differs in any important respect from that of the charged basidium.

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* One may sometimes see in sections of living material, mounted in water, that the immature spores contain alveolate cytoplasm, but careful observation will show that this is a morbid effect caused by regurgitation of the spore contents into the basidium as it is dying.

The second point implies:

(*a*) a relation between the total-spore-volume and the parent basidium volume;
(*b*) a relation between the single-spore-volume and the number of spores per basidium;
(*c*) as a corollary, the equality of the spores on the basidium.

I will consider the first two relations in detail in the following sections. Concerning the corollary, it may be said that it is so obviously true that it is usually overlooked. Nevertheless, it is a contrast from many Ascomycetes and, particularly, inoperculate Discomycetes in which there is often a habitual range in spore size among the eight spores of an ascus. Exceptions among basidiospores, however, are not infrequently found but they are certainly abnormal, as when one to three spores may abort in a tetrad and three to one variously oversized spores result.

SPORE-BASIDIUM-VOLUME

In a general way it can be seen by inspection that small-spored genera have small basidia, as *Merulius*, *Hydnum*, *Tricholoma* and *Phallus*; similarly, large-spored genera have large basidia, as *Coniophora*, *Ramaria*, *Aleurodiscus*, *Oudemansiella* and *Hymenogaster*. For more detailed study I have taken *Clavaria* and its allies, which I have had occasion to monograph in the last few years.

Spore-Basidium-Volume graph (Fig. 5, *SBV*-graph). This graph is constructed from the data in Table 1. For the reasons given in my earlier paper (1947), I have used only the more accurate measurements of basidia and have taken the mean of the size range as the average value for both spores and basidia.

Note on spore volume. I have calculated the volume of the spore as that of a sphere or an ellipsoid: thus, volume $= 0{\cdot}5236Dd^2$, where D is the spore length and d the spore width.

For elongate subcylindric spores, it would be better to regard the ends of the spores as equal hemispheres and the intervening part as a cylinder, thus

$$\text{volume} = 0{\cdot}5236d^3 + 0{\cdot}7854d^2\ (D-d).$$

Note on basidium volume. I have taken the volume of the basidium as that of a cylinder of the same length (l) but of diameter equal to the mean diameter of the basidium: this is, half the sum of the greatest diameter (w) and the smallest (w_0) at the base of the basidium. The smallest diameter is the same as the width of the uninflated subhymenial hyphae, $1{\cdot}5$–$4\,\mu$ wide, mostly $2{\cdot}5\,\mu$, and, where these details are not known, I have used the value for the most nearly related species which I have studied. Careful examination of camera-lucida drawings on squared paper gives better results, as shown on p. 38, but in this preliminary study there is no justification for great accuracy in calculation. The best method would be to treat the basidium as a figure of revolution about its long axis. Its area could be found with a planimeter from a camera-lucida drawing of a median longitudinal section and its radius of centre of gravity could be found geometrically: alternatively, its volume could be calculated from the equation to its longitudinal outline, but I have not yet satisfactorily evaluated the form of its curvature.

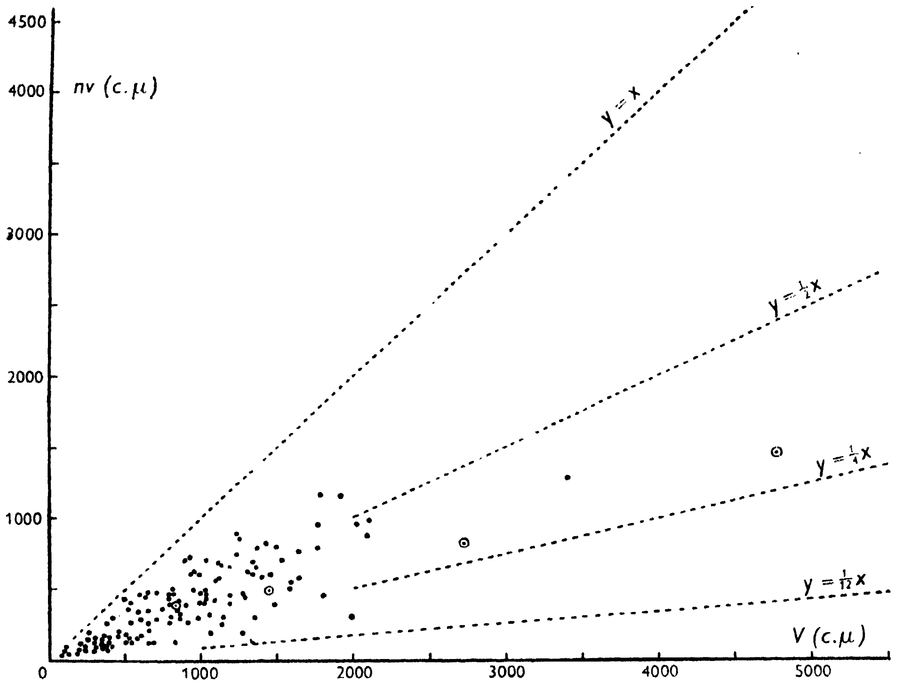

Fig. 5. To show the relation between total-spore-volume (*nv*) and basidium volume (*V*) in *Clavaria* and its allies (data from Table 1). White circles indicate species 1–4 in Table 1, which represent the genus *Clavariadelphus*. The outlying point on the right is species 111, related to *Pterula*.

Table 1. *Size and volume of spores and basidia in Clavarioid fungi (species arranged in natural groups)*

Species	Spores				Basidium		Deficit $V - nv$ (c.μ)	V/nv
	Mean size (μ)	Vol. (v) (c.μ)	No. of spores (n)	nv (c.μ)	Mean size (μ)	Vol. (V) (c.μ)		
1	2	3	4	5	6	7	8	9
Clavaria:								
C. pistillaris (1)	13·0 × 7·3	363	4	1453	105·0 × 12·0	4763	3310	3·2
C. truncata (2)	11·0 × 6·0	207	4	828	60·0 × 12·0	2722	1894	3·4
C. ligula (3)	11·5 × 4·5	122	4	488	53·0 × 7·5	1449	961	3·0
C. juncea (4)	9·0 × 4·5	95	4	380	34·0 × 8·0	838	458	2·2
C. cyanocephala (5)	13·4 × 7·9	438	2	876	65·0 × 10·0	2090	1214	2·5
C. Zippelii (6)	13·3 × 7·1	351	2	702	45·0 × 8·0	1030	328	1·4
var. (Malaya) (7)	8·5 × 5·5	135	3–4	540	53·0 × 9·5	1601	1061	3·0
C. nigrescens (8)	15·5 × 6·5	343	2	686	45·0 × 8·5	1109	423	1·6
C. fragillima (9)	13·0 × 6·0	245	(1 –) 4	980	58·0 × 10·5	2106	1126	2·0
C. Strasseri (10)	15·0 × 5·5	237	4	948	65·0 × 9·0	1776	828	1·9
C. flava (11)	15·0 × 5·3	221	4	884	50·0 × 8·5	1231	347	1·4
C. botrytis (12)	15·0 × 5·0	196	4	784	65·0 × 9·0	1776	992	2·3
C. decolorans (13)	15·0 × 5·0	196	4	784	60·0 × 8·0	1374	590	1·8
C. formosa (14)	13·0 × 5·2	184	4	736	50·0 × 8·5	1231	495	1·7
C. Mairei (15)	12·6 × 5·1	172	4	688	63·0 × 7·5	1338	650	2·0
C. aurea (16)	12·8 × 5·0	168	4	672	60·0 × 7·0	1132	460	1·7
C. Bataillei (17)	13·5 × 4·5	143	4	572	78·0 × 7·5	1657	1085	2·9
C. fennica (18)	10·3 × 4·8	124	4	496	45·0 × 6·5	814	318	1·6
C. cedretorum (19)	10·5 × 4·8	124	4	496	75·0 × 7·5	1593	1097	3·2
var. *marocana* (20)	9·0 × 5·0	118	4	472	45·0 × 8·0	1030	558	2·1

Table I (*continued*)

Species	Spores				Basidium		Deficit $V - nv$ (c.μ)	V/nv
	Mean size (μ)	Vol. (v) (c.μ)	No. of spores (n)	nv (c.μ)	Mean size (μ)	Vol. (V) (c.μ)		
I	2	3	4	5	6	7	8	9
C. fumigata (21)	10·5 × 4·6	116	4	464	40·0 × 7·3	785	321	1·7
Clavaria sp. (Malaya) (22)	9·5 × 4·8	112	4	448	60·0 × 9·5	1811	1363	4·0
Clavaria sp. (Malaya) (23)	9·3 × 4·8	110	4	440	60·0 × 8·5	1478	1038	3·4
C. stricta (24)	8·5 × 4·5	90	4	360	35·0 × 8·0	743	383	2·0
C. Invalii (25)	8·3 × 4·3	80	4	320	38·0 × 7·5	807	487	2·5
C. conjuncta (26)	10·5 × 3·8	77	4	308	83·0 × 5·5	1150	842	3·7
Clavaria sp. (Malaya) (27)	7·0 × 4·5	74	4	296	55·0 × 8·5	1355	1059	4·5
Clavaria sp. (Malaya) (28)	7·0 × 4·5	74	4	296	43·0 × 8·0	985	689	3·0
Clavaria sp. (Malaya) (29)	7·0 × 4·5	74	4	296	35·0 × 8·5	862	566	2·9
C. suecica (30)	9·3 × 3·8	70	4	280	40·0 × 6·3	608	328	2·2
C. apiculata (31)	6·5 × 4·3	63	4	252	63·0 × 6·8	1140	888	4·6
C. ochraceovirens (32)	7·5 × 3·7	54	4	216	40·0 × 6·5	664	448	3·0
C. flaccida (33)	6·6 × 3·5	43	4	172	25·0 × 5·8	363	191	2·1
C. crispula (34)	6·3 × 3·5	40	4	160	30·0 × 5·3	358	146	2·3
C. gracilis (35)	5·7 × 3·3	33	4	132	35·0 × 5·8	508	376	3·9
Clavaria sp. (Malaya) (36)	5·8 × 3·3	33	4	132	50·0 × 6·5	831	699	6·4
C. Bourdotiana (37)	5·0 × 3·0	24	4	96	23·0 × 4·8	234	138	2·3
C. rugosa (38)	11·2 × 9·0	475	2	950	63·0 × 7·8	2026	1075	2·0
Clavaria sp. (Malaya) (39)	11·0 × 10·0	581	2	1162	68·0 × 8·0	1923	761	1·6
Clavaria sp. (Malaya) (40)	9·0 × 9·0	382	2	764	50·0 × 9·0	1659	895	2·2
C. ornatipes (41)	9·3 × 8·5	352	2	704	28·0 × 6·8	900	200	1·3
C. cristata (42)	9·0 × 8·3	325	2	650	50·0 × 7·0	1188	538	1·8
C. cinerea (43)	9·0 × 8·0	302	2	604	55·0 × 8·5	1341	737	2·2
Clavaria sp. (Malaya) (44)	9·0 × 8·0	302	2	604	31·0 × 6·3	936	332	1·6
*C. Léveillé*i (45)	9·0 × 8·0	302	2	604	40·0 × 7·5	985	381	1·7
Clavaria sp. (Malaya) (46)	9·0 × 7·8	287	2	574	47·0 × 6·5	1116	542	2·0
C. cartilaginea (47)	8·5 × 7·3	237	2	474	40·0 × 6·5	1039	565	2·2
C. amethystina (48)	9·0 × 7·0	231	2	462	51·0 × 6·0	1001	539	2·2
Clavaria sp. (Malaya) (49)	7·8 × 7·0	200	2	400	47·0 × 6·5	998	598	2·5
C. surculus (50)	14·5 × 4·0	122	4	488	40·0 × 8·0	950	462	1·9
Clavaria sp. (Malaya) (51)	7·5 × 5·5	119	4	476	39·0 × 6·5	665	189	1·4
C. luteo-alba var. (52)	8·0 × 5·0	105	4	420	46·0 × 8·0	1092	672	2·6
C. fuscata (53)	8·5 × 4·8	100	2	200	26·0 × 7·0	461	261	2·3
C. luteo-tenerrima (54)	7·5 × 4·5	80	4	320	50·0 × 7·5	1062	742	3·3
Clavaria sp. (Malaya) (55)	6·3 × 4·8	76	4	304	60·0 × 10·0	1991	1687	6·6
C. pulchra (56)	6·3 × 4·5	67	4	268	55·0 × 5·5	784	516	2·9
C. Pullei (57)	5·7 × 4·3	55	2	110	28·0 × 5·8	374	264	3·4
C. affinis (58)	6·5 × 3·5	42	4	168	32·0 × 6·0	402	234	2·4
C. luteo-alba (59)	6·5 × 3·5	42	4	168	43·0 × 5·5	613	445	3·6
C. propera (60)	6·5 × 3·5	42	4	168	27·0 × 5·5	339	171	2·0
C. depokensis (61)	5·0 × 3·5	32	4	128	35·0 × 6·8	660	532	5·0
C. umbrinella (62)	5·0 × 3·5	32	4	128	37·5 × 6·5	678	550	5·2
C. vernalis (63)	9·5 × 2·5	31	4	124	21·5 × 4·5	207	83	1·8
C. vernalis (Donk) (64)	9·5 × 2·5	31	4	124	30·0 × 7·0	543	419	4·4
C. afflata (65)	5·5 × 2·9	23	4	46	21·0 × 4·5	174	128	3·8
Clavaria sp. (Malaya) (66)	4·5 × 3·0	21	4	84	25·0 × 5·5	356	272	4·2
Clavaria sp. (Malaya) (67)	5·0 × 2·8	20	4	80	23·0 × 5·5	289	209	3·6
C. biformis (68)	3·8 × 2·9	17	4	68	22·5 × 4·5	181	113	2·7
C. fusiformis (69)	7·0 × 6·5	155	4	620	53·0 × 7·5	959	339	1·5
C. Holmskjoldii (70)	6·5 × 6·5	144	4	576	53·0 × 9·0	1401	825	2·3
C. corniculata (71)	5·8 × 5·8	100	4	400	48·0 × 8·0	1020	620	2·6
C. miniata (72)	6·0 × 5·5	95	4	380	65·0 × 7·8	1489	1109	3·9
C. spiralis (73)	6·3 × 5·5	100	4	400	50·0 × 8·0	1188	788	3·0
C. amoena (74)	5·5 × 5·3	81	4	324	48·0 × 7·5	868	544	2·7
C. globospora (75)	5·5 × 5·5	87	2	174	63·0 × 4·5	606	432	3·6
C. griseola (76)	5·5 × 5·5	87	4	348	33·0 × 7·5	648	300	2·0
C. alcicornis (77)	5·5 × 4·8	66	4	264	43·0 × 8·5	913	647	3·5
C. cineroides (78)	5·0 × 5·0	65	4	260	43·0 × 6·3	654	394	2·5
C. dichotoma (79)	5·0 × 4·3	48	4	192	27·0 × 5·0	260	68	1·3
C. subtilis (80)	5·0 × 4·0	42	4	168	33·0 × 5·3	390	222	2·3
C. luteo-ochracea (81)	4·5 × 3·5	29	4	116	30·0 × 5·5	340	224	2·8
C. tenuicula (82)	3·25 × 2·75	13	4	52	16·0 × 4·25	122	70	2·3
C. minutula (83)	2·75 × 2·65	10	4	40	17·0 × 4·25	137	97	3·4
Clavaria sp. (Malaya) (84)	10·0 × 9·0	424	2	848	53·0 × 8·5	1259	411	1·5
C. acuta (85)	8·5 × 7·0	218	2	436	33·0 × 9·0	784	348	1·8

Table 1 (*continued*)

Species	Spores				Basidium		Deficit $V-nv$ (c.μ)	V/nv
	Mean size (μ)	Vol. (v) (c.μ)	No. of spores (n)	nv (c.μ)	Mean size (μ)	Vol. (V) (c.μ)		
1	2	3	4	5	6	7	8	9
C. argillacea (86)	10·5 × 5·3	155	4	620	67·0 × 7·0	1315	695	2·1
var. *obtusata* (87)	9·3 × 5·8	164	4	654	69·5 × 7·0	1364	710	2·1
var. *obtusata* (88)	9·5 × 5·5	151	4	604	55·0 × 8·5	1453	849	2·4
var. *sphagnicola* (89)	10·3 × 6·2	207	4	828	67·5 × 7·5	1434	606	1·7
C. helicoides (90)	10·5 × 4·8	124	4	496	45·0 × 9·0	1272	776	2·5
C. straminea (91)	6·0 × 6·0	113	4	452	50·0 × 8·0	1188	736	2·6
C. incarnata (92)	8·3 × 5·0	109	4	436	53·0 × 8·0	1040	604	2·4
C. Zollingeri (93)	5·5 × 4·0	46	4	184	50·0 × 7·5	1062	878	6·0
C. occidentalis (94)	7·0 × 3·5	45	4	180	65·0 × 8·0	1276	1096	7·0
C. fumosa (95)	6·5 × 3·5	42	4	168	38·0 × 6·5	526	358	3·0
Clavaria sp. (Malaya) (96)	4·8 × 4·0	40	4	160	27·0 × 6·5	411	251	2·6
C. vermicularis (97)	6·0 × 3·5	39	4	156	38·0 × 6·8	578	422	3·6
C. Corbierei (98)	3·8 × 3·5	24	4	96	16·5 × 3·8	108	12	1·1
C. nebulosoides (99)	5·5 × 2·5	18	4	72	30·0 × 5·0	289	217	4·0
Clavaria sp. (Malaya) (100)	5·8 × 2·4	17	4	68	20·0 × 6·0	304	236	4·5
C. nigrita (101)	5·0 × 2·25	13	4	52	23·0 × 5·5	289	237	5·5
C. Guilleminii (102)	4·0 × 2·5	13	4	52	21·0 × 4·0	128	76	2·5
C. Kunzei (103)	4·3 × 3·4	26	4	104	33·0 × 5·5	415	311	4·0
C. pulchella (104)	3·8 × 3·0	18	4	72	25·0 × 6·5	380	308	5·4
C. angulispora (105)	6·3 × 3·75	46	4	184	28·0 × 6·3	507	323	2·8
C. semivestita (106)	17·0 × 6·0	321	4	1284	60·0 × 13·0	3405	2121	2·7
Clavaria sp. (Malaya) (107)	9·3 × 6·3	193	2	386	28·0 × 9·0	792	406	2·0
C. dendroides Jungh. (108)	9·8 × 8·8	397	2	794	45·0 × 10·5	1493	699	1·9
Clavaria sp. (Malaya) (109)	13·0 × 5·5	206	2	412	36·0 × 9·0	855	443	2·0
C. javanica (110)	4·25 × 3·25	25	4	100	14·0 × 3·5	113	13	1·1
Pterula sp. (Malaya) (111)	13·0 × 13·0	1150	4	4600	68·0 × 16·5	5117	517	1·1
P. xylogena (112)	12·0 × 6·8	291	4	1164	50·0 × 11·5	1788	624	1·5
Pterula sp. (Malaya) (113)	12·5 × 4·5	133	2	266	21·0 × 7·5	372	106	1·4
Pterula sp. (Malaya) (114)	12·0 × 3·5	77	2	154	24·0 × 6·5	340	186	2·2
Pterula sp. (Malaya) (115)	6·5 × 3·0	31	4	124	21·0 × 5·5	232	108	1·9
P. gracilis (116)	11·5 × 6·0	217	2	434	25·0 × 8·0	490	56	1·1
Pterula sp. (Malaya) (117)	6·5 × 3·3	37	4	148	25·0 × 6·5	355	207	2·4
Pterula sp. (Malaya) (118)	11·0 × 4·5	117	4	468	25·0 × 9·0	594	126	1·3
P. multifida (119)	6·0 × 3·5	39	4	156	27·0 × 5·5	298	142	2·0
Pterula sp. (Malaya) (120)	9·5 × 4·5	101	4	404	27·0 × 8·0	530	126	1·3
var. (Malaya) (121)	9·0 × 4·0	75	4	300	21·0 × 8·0	412	112	1·4
Pterula sp. (Malaya) (122)	6·5 × 3·3	37	4	148	21·0 × 6·0	258	110	1·7
Typhula:								
T. phacorrhiza (123)	15·0 × 6·0	283	2	566	27·0 × 10·5	1099	533	2·0
T. pertenuis (124)	9·0 × 5·0	118	4	472	25·0 × 8·0	707	235	1·5
T. idahoensis (125)	10·5 × 4·5	111	4	444	30·0 × 7·0	637	193	1·5
T. athyrii (126)	11·0 × 4·0	92	4	368	30·0 × 8·0	848	480	2·3
T. viburni (127)	8·5 × 4·5	90	4	360	22·0 × 7·5	523	163	1·4
T. latissima (128)	10·5 × 4·0	88	4	352	30·0 × 8·0	848	496	2·4
T. sclerotioides (129)	8·3 × 2·8	42	4	168	25·0 × 6·0	452	284	2·6
T. erythropus (130)	7·0 × 3·0	33	4	132	24·0 × 6·0	382	250	2·9
T. pallens (131)	6·5 × 3·0	31	4	124	23·0 × 5·0	289	165	2·3
Pistillaria:								
P. ampelina (132)	9·0 × 7·0	231	2	462	27·0 × 9·5	815	353	1·8
P. micans (133)	10·5 × 6·0	198	2	396	35·0 × 8·0	831	435	2·1
P. quisquiliaris (134)	12·5 × 5·25	180	4	720	35·0 × 8·5	925	205	1·3
P. quisquiliaris (135)	13·0 × 5·0	170	2	340	35·0 × 6·3	582	242	1·7
P. capitata (136)	9·8 × 4·3	95	4	380	30·0 × 9·0	906	526	2·4
P. setipes (137)	9·0 × 3·8	66	4	264	27·0 × 7·0	530	266	2·0
P. uncialis (138)	6·5 × 2·5	21	4	84	33·0 × 4·5	317	233	3·8
P. uncialis (139)	4·5 × 2·75	18	4	72	24·0 × 4·5	231	159	3·2
P. aculeata (140)	5·0 × 2·5	16	4	64	12·0 × 4·3	97	33	1·5
P. mucedinacea (141)	6·0 × 1·75	9	4	36	11·0 × 4·0	78	42	2·2
P. hyalina (142)	8·5 × 4·0	71	4	284	50·0 × 6·5	693	409	2·5

The following conclusions can be drawn from this graph:

(i) among Clavarioid fungi there are no large basidia with small spores or small basidia with large spores;

(ii) for most species the proportion of basidium volume to total-spore-volume lies between 2:1 and 4:1;

(iii) there is no suggestion of a lower ratio than 1:1.

The conclusions can hold only if the total-spore-volume bears a direct relation to that of the basidium, and the only physiological means of securing this is to pass a definite proportion of the basidium contents into the spores. If the basidium were just a vehicle supporting the spores and passing material into them from the subhymenial hyphae, no such relation could be expected: likewise, if the spores merely absorbed a variable part of the basidium contents. The graph, therefore, supports the hypothesis that the spores are simply forced out of the basidium.

The ratios 4 : 1 and 2 : 1, on the other hand, imply that there is a loss of protoplasm in the formation of the spores, because observation shows that the basidium empties itself except for a thin lining layer of cytoplasm and a small residuum in the sterigmata, neither of which can account for so large a discrepancy. The explanation lies with the initial vacuolation of the charged basidium. The total volume of the vacuoles in the full-sized basidium must be subtracted from the volume of the basidium to give that of the dense protoplasm. Thus, if V is the volume of the full-sized basidium, A the volume of the vacuoles in it, v the volume of a spore, and n the number of spores on the basidium, one would expect, roughly,

$$V-A=nv, \quad \text{or} \quad A=V-nv,$$

for the residual cytoplasm in the sterigmatic tips and lining layer of the basidium are negligible compared with V.

Now, it is easy to obtain data for V and v, but it is commonly very difficult to find such for A, yet a knowledge of A is indispensable for the comparison of basidia of different sizes and of different species, genera, families and so on. To avoid this quantity, therefore, I regard the proportion of V to nv as the *index of initial vacuolation of the full-sized basidium*, which I call i, the final vacuolation being complete; thus

$$V/nv=i \quad \text{and} \quad V=inv.$$

The greater the value of i, the greater will be the discrepancy between V and nv, or between basidium and spore volume. Thus, if $i=1$, the full-grown basidium will not be vacuolate and practically all its contents will pass into the spores which will be large compared with the basidium. If $i=4$, three-quarters of the full-grown basidium will consist of vacuole and the spores will be small compared with the basidium. The figures in the column 'Deficit' in Table 1 refer to initial vacuolation rather than to residual cytoplasm as, at first, one would imagine.

To test this deduction, and to obtain more accurate results, I endeavoured to study camera-lucida drawings of full-sized basidia with clearly defined vacuoles. Unfortunately the *Clavaria* basidium is not a suitable object for this purpose because of its long and usually flexuous stalk, and its irregular vacuolation. It can be seen, however, by inspection that this stalk is more or less wholly vacuolate and that the charging is incomplete, being mainly in the clavate distal half, third or quarter of the basidium, so that one would expect i to lie between 2 and 4. As suitable material I managed to find only one *Clavaria*, but I have obtained corroboration from four agarics, the basidia of which are more easily extricated for measurement, are more regular in shape and have sharp, spherical or ellipsoid vacuoles when full sized.

Volumes of spores, basidia and vacuoles. In Tables 2–6 I have arranged for three species of *Oudemansiella*, an *Amanita* and a *Clavaria*, the actual data obtained from camera-lucida drawings of regular and distinctly vacuolate full-sized basidia.

Table 2. *Basidium size, initial vacuolation and spore size in* Oudemansiella Canarii

	Basidium		Sterigmata		Vacuoles total volume (A) (c. μ)	$V-(A-S)$ (c. μ)	i	d (calculated) (μ)
	Size (μ) $l \times w \times w_0$	Volume (V) (c. μ)	Size (μ)	Volume (S) (c. μ)				
	1	2	3	4	5	6	7	8
(i)	81·5 × 27·0 × 10·0	26,694	—	—	3210	23,484	1·137	22·39
(ii)	96·5 × 27·5 × 9·0	26,240	—	—	2594	23,646	1·110	22·44
(iii)	80·0 × 27·5 × 7·5	25,119	9·0 × 5·7	306	155	24,813	(1)	22·80
(iv)	80·0 × 26·0 × 6·5	20,360	13·5 × 7·2	733	573	19,627	(1)	21·08
(v)	84·0 × 23·0 × 7·0	19,485	—	—	4227	15,258	1·277	19·38
(vi)	83·5 × 21·5 × 6·5	17,916	—	—	4393	13,523	1·325	18·62
(vii)	98·5 × 20·3 × 8·0	17,348	—	—	5650	11,698	1·483	17·74
(viii)	62·0 × 23·0 × 6·0	16,903	7·0 × 5·0	183	3217	13,686	1·235	18·69
(ix)	80·0 × 21·0 × 7·5	16,885	—	—	5848	11,037	1·530	17·44
(x)	63·5 × 21·0 × 6·0	15,419	8·3 × 5·5	263	3506	11,913	1·294	17·84
(xi)	64·0 × 21·0 × 6·5	14,140	—	—	3787	10,353	1·366	17·03

Range of d (calculated), 17·0–22·8 μ.
Range of d (observed), 16·0–23·0 μ.
Average value of i (excluding basidia (iii) and (iv)) = 1·306.

Table 3. *Basidium size, initial vacuolation and spore size in* Oudemansiella radicata

	Basidium		Sterigmata		Vacuoles total volume (A) (c. μ)	$V-(A-S)$ (c. μ)	i	d (calculated) (μ)
	Size (μ) $l \times w \times w_0$	Volume (V) (c. μ)	Size (μ)	Volume (S) (c. μ)				
	1	2	3	4	5	6	7	8
(i)	60·5 × 20·0 × 5·5	9427	1·7 × 3·75	25	868	8584	1·098	16·01
(ii)	52·7 × 18·8 × 6·0	8112	5·1 × 4·5	108	1140	7080	1·146	15·01
(iii)	59·5 × 18·8 × 5·5	8079	—	—	1139	6940	1·164	14·90
(iv)	49·8 × 19·5 × 5·0	7549	—	—	295	7254	1·041	15·13
(v)	50·0 × 18·2 × 5·0	6137	—	—	393	5744	1·069	14·00

Range of d (calculated), 14·0–16·0 μ.
Range of d (observed), 10·5–16·0 μ.
Average value of i = 1·104.

The presence of the hyaline cap was taken to indicate the full size of the basidium, or the presence of emerging sterigmata, in which case the volume of the sterigmata had to be subtracted from that of the vacuole to give the value of A when the basidium was full sized, but without sterigmata. The sterigmata were treated as cones and their volume estimated accordingly. In column 7, the value of i is given as calculated from the figures in columns 2 and 6, that is, as $V : V-A$.

Table 4. ***Basidium size, initial vacuolation and spore size in*** Oudemansiella *sp*. C. (=Mycena illuminans *P. Henn.*)

	Basidium		Sterigmata		Vacuoles total volume (A) (c. μ)	$V-(A-S)$ (c. μ)	i	d (calculated) (μ)
	Size (μ) $l \times w \times w_0$	Volume (V) (c. μ)	Size (μ)	Volume (S) (c. μ)				
	1	2	3	4	5	6	7	8
(i)	37·0 × 16·3 × 4·0	3144	2·5 × 3·0	24	436	2732	1·151	10·91
(ii)	38·5 × 15·0 × 4·5	2787	0·6 × 2·5	4	903	1888	1·479	9·65
(iii)	37·7 × 15·5 × 3·0	2755	2·3 × 2·5	15	798	1972	1·397	9·79
(iv)	38·5 × 14·5 × 5·5	2749	5·7 × 3·7	82	795	2036	1·350	9·90
(v)	34·5 × 14·5 × 4·5	2533	6·0 × 3·2	64	693	1904	1·330	9·68
(vi)	36·0 × 13·5 × 3·5	2290	5·8 × 3·2	62	697	1655	1·384	9·24
(vii)	34·5 × 14·2 × 3·0	2074	3·3 × 2·9	29	613	1488	1·395	8·92
(viii)	32·0 × 13·5 × 5·0	2038	0·5 × 2·7	5	765	1278	1·594	8·48
(ix)	31·0 × 13·4 × 3·5	1908	—	—	349	1559	1·224	9·06
(x)	24·0 × 13·0 × 3·5	1701	—	—	266	1453	1·185	8·81

Range of d (calculated), 8·5–10·9 μ.
Range of d (observed), 8·2–11·0 μ.
Average value of $i = 1{\cdot}349$.

Table 5. ***Basidium size, initial vacuolation and spore size in*** Amanita *sp*. (*aff*. A. adnata *W.G. Sm.*)

	Basidium		Sterigmata		Vacuoles total volume (A) (c. μ)	$V-(A-S)$ (c. μ)	i	d (calculated) (μ)
	Size (μ) $l \times w \times w_0$	Volume (V) (c. μ)	Size (μ)	Volume (S) (c. μ)				
	1	2	3	4	5	6	7	8
(i)	40·0 × 11·5 × 4·2	2316	5·0 × 2·0	21	129	2208	1·049	10·17
(ii)	40·0 × 10·0 × 4·5	2242	4·5 × 2·0	19	229	2032	1·104	9·90
(iii)	46·8 × 10·6 × 4·5	2094	6·0 × 2·7	46	468	1672	1·253	9·28
(iv)	34·5 × 10·7 × 5·0	1734	4·4 × 2·5	29	481	1282	1·352	8·49

Range of d (calculated), 8·5–10·2 μ.
Range of d (observed), 7·0–9·5 μ.
Average value of $i = 1{\cdot}190$.

Table 6. ***Basidium size, initial vacuolation and spore size in*** Clavaria amoena

	Basidium		Sterigmata		Vacuoles total volume (A) (c. μ)	$V-(A-S)$ (c. μ)	i	d (calculated) (μ)
	Size (μ) $l \times w \times w_0$	Volume (V) (c. μ)	Size (μ)	Volume (S) (c. μ)				
	1	2	3	4	5	6	7	8
(i)	53·0 × 9·8 × 3·0	1704	—	—	852	852	2·00	7·41
(ii)	42·5 × 9·2 × 2·5	1153	7·5 × 2·5	49	493	709	1·63	6·97
(iii)	38·0 × 8·0 × 2·5	822	7·5 × 2·5	49	352	519	1·59	6·28

Range of d (calculated), 6·3–7·4 μ.
Range of d (observed), 4·5–7·2 μ.
Average value of $i = 1{\cdot}74$.

Now, these fungi have globose or subglobose spores and so it is possible to calculate what sized spores may be expected from the individual basidia (for which purpose I made drawings of large, medium and small basidia for each species). Thus, if d is the diameter of the spore, its volume is kd^3, where $k=\pi/6$, and assuming

$$V-A=nkd^3,$$

then

$$d=\sqrt[3]{\frac{V-A}{nk}},$$

or

$$d=\sqrt[3]{\frac{V-A}{2{\cdot}0944}} \quad \text{when } n=4.$$

Thus, in column 8 of Tables 2–6, I have shown the calculated value of the spore for each basidium (the species having 4-spored basidia), and at the foot of each table I have compared the range of d (calculated) with that actually observed in a spore print from the same fruit body as that from which the drawings of basidia were taken. The agreement is remarkably close, especially for the large spores and basidia of *Oudemansiella*, and one must conclude that the enlarging basal vacuole in the basidia of these fungi scours the basidium of all but the thinnest layer of cytoplasm in its apical progress: from observation, all but the extreme tips of the sterigmata become vacuolate.

Table 7. *Volumes of spores and basidia in* Hygrophorus *with dimorphous spores*

	Spore volume (v) (c. μ)	n	nv (c. μ)	Basidium volume (V) (c. μ)	$V:nv$
H. firmus:					
Large spores	530	4	2120	4620	2·2
Small spores	54	4	216	565	2·6
H. hypohaemactus:					
Large spores	265	4	1060	1500	1·4
Small spores	25	4	100	272	2·7

Ratio of v (large spores) to v (small spores) in *H. firmus*, 9·8.
Ratio of v (large spores) to v (small spores) in *H. hypohaemactus*, 10·6.
Ratio of V (large basidia) to V (small basidia) in *H. firmus*, 8·2.
Ratio of V (large basidia) to V (small basidia) in *H. hypohaemactus*, 5·5.

In two basidia of *O. Canarii* ((iii) and (iv) in Table 2), there seems to have been no initial vacuolation: indeed, the volume of their rudimentary sterigmata exceeds that of the visible vacuoles. Similarly, Buller's figures of the basidium of *Coprinus sterquilinus* and mine of *Hygrophorus firmus* (Corner, 1936) suggest that there may be no initial vacuolation in some agaric basidia. I think the conclusion is wrong. The initial vacuole of the completely charged basidium does not necessarily begin as one or a few basal vacuoles but develops from the confluence of very small vacuoles dispersed through the basidium. Somehow these vacuoles migrate to the base of the basidium whence they push the cytoplasm to the apex. Thus, one may overlook the earlier stages of initial vacuolation. Calculations from $V:nv$ for *Hygrophorus firmus* suggest a value of 2 for its index of initial vacuolation.

Hygrophorus ***with dimorphous spores.*** I refer to *H. firmus* and *H. hypohaemactus* which I studied in Malaya (1936). On the same fruit body they have large basidia bearing large spores and small basidia bearing small spores. As shown in Table 7, the large spores have ten times the volume of the small spores (both being produced in tetrads). In *H. firmus*, too; the large basidia have roughly ten times the volume of the small basidia (as calculated from the mean sizes of the basidia), but in *H. hypohaemactus* the large basidia are only five times the size of the small, apparently because of the low initial vacuolation of its large basidia.

Table 8. *Spore number and mean spore size in Agarics*

Species	Dispores ($n=2$)		Tetraspores ($n=4$)		Authority
	Size (μ)	Volume (c. μ)	Size (μ)	v = volume (c. μ)	
Mycena:					
M. alcalina	11·5 × 7·3	321·0 = 2·3*v*	9·0 × 5·5	143·0	Smith (1934)
M. capillaris	—	285·0 = 2·0*v**	—	143·3	,,
M. capillaris	12·0 × 5·5	190·0 = 2·0*v*	9·0 × 4·5	95·0	,,
M. citrino-marginata†	—	255·2 = 1·3*v**	—	196·6	,,
M. citrino-marginata†	13·0 × 5·5	206·0 = 1·3*v*	10·0 × 5·5	158·0	,,
M. clavicularis	11·0 × 5·5	174·0 = 1·8*v*	9·0 × 4·5	95·0	,,
M. dissiliens†	7·5 × 7·0	192·0 = 1·6*v*	7·5 × 5·5	119·0	,,
M. epipterygia	11·0 × 7·0	282·0 = 1·3*v*	10·0 × 6·5	221·0	,,
M. graveolens	9·0 × 4·75	105·0 = 1·6*v*	7·5 × 4·0	64·0	,,
M. immaculata	9·0 × 2·85	38·0 = 1·3*v*	7·5 × 2·75	30·0	,,
M. lactea	8·0 × 6·75	185·0 = 3·8*v*	7·5 × 3·5	48·0	,,
M. leptocephala†	12·5 × 5·75	210·0 = 2·3*v*	8·5 × 4·5	90·0	,,
M. metata	8·5 × 4·5	90·0 = 1·9*v*	7·5 × 3·5	48·0	,,
M. polygramma var. *albida*†	10·0 × 6·5	221·0 = 1·4*v*	9·0 × 5·7	155·0	,,
M. roseipallens	7·5 × 5·5	119·0 = 3·0*v*	6·5 × 3·5	40·0	,,
M. supina	9·5 × 7·5	280·0 = 1·3*v*	7·5 × 7·5	210·0	,,
M. viscosa	10·0 × 7·25	280·0 = 2·1*v*	8·5 × 5·5	135·0	,,
Hygrophorus:					
H. conicus	13·75 × 7·5	405·0 = 2·1*v*	11·0 × 5·75	190·0	Maire (1926)
H. virgineus	13·0 × 5·75	225·0 = 1·6*v*	9·0 × 5·5	143·0	,,
Inocybe:					
I. hirtella	11·25 × 6·5	243·0 = 1·2*v*	10·5 × 6·0	198·0	,,
Galera:					
G. tenera	14·25 × 8·25	508·0 = 1·7*v*	11·4 × 7·0	293·0	Sass (1929)
Naucoria:					
N. semi-orbicularis	14·5 × 9·0	615·0 = 1·6*v*	12·25 × 7·7	380·0	,,
Coprinus:					
C. ephemerus	12·75 × 6·5	282·0 = 1·5*v*	10·6 × 6·7	195·0	Lange, Rea.
C. ephemerus	13·5 × 9·25	605·0 = 1·6*v*	13·0 × 7·5	383·0	Sass (1929)
Agaricus:					
A. campestris	7·0 × 5·4	109·0 = 0·74*v*	8·4 × 5·8	148·0	Cayley (1936)
Boletus:					
B. tesselatus	19·5 × 6·75	465·0 = 2·2*v*	13·5 × 5·5	214·0	Maire (1926)

* Figures given by Smith.
† Often 3-spored as well as 2-spored.

SPORE SIZE AND SPORE NUMBER

If $V = inv$, where V is the volume of the basidium, v is that of a spore, n is the number of spores per basidium, and i is the index of initial vacuolation, then it follows that variations in n will cause variations in v, provided V and i are constant. Similarly, V will vary if v and i are constant. General observations on species with 2- 3- and 4-spored basidia in the same or different fruit bodies show that the size of the basidium remains roughly constant but that spore size alters for differences in their number. In Tables 8 and 9 I have collected published data on the variation of spore size with number, as well as some of my own, and, on the assumption that V and i remain constant for a species, I have related the variation of v with n and expressed the values of v for differences in

n as multiples of $v = 1$ when $n = 4$, as the usual state, or when $v = 2$ for normally disporous species.

Except for Cayley's observation on *Agaricus campestris*, there is a general agreement between the values of v as calculated from observed spore size and the theoretical value according to n. Errors arise from the method of calculation of spore volume and from the presence of residual cytoplasm in 2-spored basidia. Regarding the first source, I have assumed the spore to be an ellipsoid for the purpose of simplicity and consistency of method: actually the spores may be pip-shaped or subcylindric and, as Smith observed, dispores of a species are usually more or less differently shaped from tetraspores. Regarding the second source, one can often see the residual cytoplasm in the sterigmata and as an apical cap to the basidium. Thus, in *Typhula hyalina*, actual measurements of vacuoles in four full-sized basidia gave $i = 1{\cdot}5$ ($1{\cdot}3$–$1{\cdot}7$), but the ratio of $V : nv$ is 2: observation shows a considerable apical cap of residual cytoplasm in discharged basidia and a greater residuum in 1-spored than in 2-spored basidia. In *T. hyalina*, too, I carefully checked that the 1-spored basidia were of the same size as the 2-spored.

Table 9. *Spore number and average spore size in Thelephoraceae*

Species	Monospores		Dispores		Trispores	
	Size (μ)	Volume (c. μ)	Size (μ)	Volume (c. μ)	Size (μ)	Volume (c. μ)
Theoretical values ...	—	$4v$	—	$2v$	—	$1{\cdot}33v$
Craterellus:						
C. cornucopioides	—	—	13·0 × 8·9	570·0	—	—
Craterellus sp. (Malaya)	—	—	—	—	9·0 × 7·2	$244{\cdot}0 = 1{\cdot}3v$
Cantharellus sp.	—	—	—	—	—	—
Cantharellus sp.	—	—	—	—	—	—
Theoretical values ...	—	$2v$	—	v	—	$0{\cdot}67v$
Stereum:						
S. aterrimum	14·0 × 11·5	$970{\cdot}0 = 1{\cdot}8v$	11·5 × 9·5	$543{\cdot}0 = v$	10·0 × 8·3	$361{\cdot}0 = 0{\cdot}66v$
Clavaria sp.	13·0 × 9·8	$654{\cdot}0 = 1{\cdot}7v$	9·0 × 9·0	$382{\cdot}0 = v$	—	—
Typhula hyalina	12·0 × 5·7	$200{\cdot}0 = 1{\cdot}7v$	9·5 × 5·0	$120{\cdot}0 = v$	—	—

Species	Tetraspores		Pentaspores		Hexaspores	
	Size (μ)	Volume (c. μ)	Size (μ)	Volume (c. μ)	Size (μ)	Volume (c. μ)
Theoretical values ...	—	v	—	$0{\cdot}8v$	—	$0{\cdot}67v$
Craterellus:						
C. cornucopioides	—	—	—	—	—	—
Craterellus sp. (Malaya)	8·8 × 6·5	$195{\cdot}0 = v$	8·2 × 6·4	$176{\cdot}0 = 0{\cdot}9v$	9·2 × 5·6	$151{\cdot}0 = 0{\cdot}77v$
Cantharellus sp.	9·3 × 6·9	$232{\cdot}0 = v$	—	—	8·8 × 5·2	$125{\cdot}0 \times 0{\cdot}54v$
Cantharellus sp.	8·2 × 5·9	$150{\cdot}0 = v$	—	—	7·1 × 5·1	$97{\cdot}0 = 0{\cdot}65v$

The most striking agreement between the observed and calculated spore sizes for differences in spore number is given by *Craterellus cornucopioides* and a very closely allied Malayan species, the two giving a range from $n = 2$ to $n = 6$. The volume of the dispore of *C. cornucopioides*, however, is nearly three times that of the tetraspore of the Malayan species. The difference from the theoretical value is too great to be ascribed to an error in observation, for I have studied both species carefully and my measurements of spores and basidia for *C. cornucopioides* agree with those of Donk (1933). The explanation is to be found in the larger basidia of *C. cornucopioides*, the mean size of which, 80 × 8·5 μ, has a volume about 1·5 times that of the tropical species (56 × 8 μ). Therefore, theoretically, one would expect the dispore of *C. cornucopioides* to be 2 × 1·5 times the size of the tetraspore of the tropical species.

The case of *Agaricus campestris* (Table 8) suggests that there must be a difference in size or initial vacuolation between the basidia of disporous and of tetrasporous fruit bodies. Buller gives the volume of abnormal monospores as double that of dispores (Buller, 1922, vol. 2, chap. 1).

In the case of *Craterellus ephemerus*, the species so named by Sass and Ricken appears to be different from that of Lange and Rea, which has *C. bisporus* Lange as its 2-spored state (Josserand, 1930).

Among the Clavarioid fungi listed in Table 1, the following pairs of allied 2-spored and 4-spored species have basidia about the same size and the dispore roughly double the volume of the tetraspore:

2-spored	4-spored
Clavaria cyanocephala (no. 5)	*Clavaria fragillima* (no. 9)
Pterula sp. (no. 114)	*Pterula* sp. (no. 115)
Pterula gracilis (no. 116)	*Pterula* sp. (no. 118)

The alternative state is shown by *Pistillaria quisquiliaris* in which the *V* varies with *n* while *v* is constant. Thus the dispores described by Donk and Lundell have almost the same volume as the tetraspores described by Josserand (Table 1, nos. 134 and 135). Accordingly, *nv* for tetraspores has double the value of *nv* for dispores, and the apparent discrepancy is reconciled by the large basidia described by Josserand for the tetrasporous fruit bodies: these larger basidia also seem to have lower initial vacuolation, as one would expect if the smaller 2-spored basidia matured at an earlier, and possibly 2-nucleate, state before the basidium was normally charged. It seems that the same relation may hold between the 2-spored state of *Clavaria nigrescens* in Europe and its 4-spored state in North America.

One must conclude that these relations between *V*, *n* and *v* in a species, or group of allied species, can hold only if the basidium is charged with a definite amount of protoplasm which it divides equally among the spores and that, once the basidium has begun to work at the time of initial vacuolation, no more protoplasm is made in it. Thus, the basidium exemplifies the *all or nothing* principle of single cell mechanism and is referable, through the sporangium, to the unicellular organism. There is no evidence that the basidiospore controls its own development unless in general shape, but, as I hope to show in a later paper, even this is doubtful.

THREE EXCEPTIONS

I have no doubt that many exceptions to the normal mechanism of the basidium will be found. The following three show how an understanding of the details of development may explain apparent anomalies and how these can be rectified by assuming a normal state.

Fomes subresinosus. Spores are difficult to find on the fruit bodies of this species in Malaya. At most, a feeble white deposit of thin-walled, hyaline, aguttate spores, *c.* 6 × 4·5 μ, can be obtained, though the basidia measure 25–45 × 9·5–14 μ. If fully charged, such basidia should produce globose spores *c.* $8{\cdot}7\,\mu$ in diameter, resembling those of ***Amauroderma*** with which genus the species is related. Examination of the living basidia in course of development shows that they never become charged with dense protoplasm, but that the vacuoles of the stage of inception continue to enlarge, and eventually expel into the spores only the small amount of protoplasm which persists at the apex of the juvenile basidium, cf. Fig. 6. In other words, the initial vacuolation of the full-sized basidium is exceptionally high, *c.* 5·7. Thus:

Mean size of basidium $= 35 \times 10{\cdot}7 \times 3{\cdot}5\,\mu = 1386$ c.μ (V).
Mean size of sterigmata $= 4 \times 1{\cdot}5\,\mu = 2{\cdot}36$ c.μ (s).
Mean size of spore $= 5{\cdot}8 \times 4{\cdot}5\,\mu = 61$ c.μ (v).

Mean volume of protoplasm at the apex of the full-sized basidium is *c.* 270 c.μ.

$$n\,(v+s) = 4\,(61 + 2{\cdot}36)\ \text{c.}\mu = 253{\cdot}44\ \text{c.}\mu.$$

And, if the basidium were fully charged and produced globose spores of diameter d, then $d^3 = 6V/\pi$, whence $d = 8{\cdot}7\,\mu$.

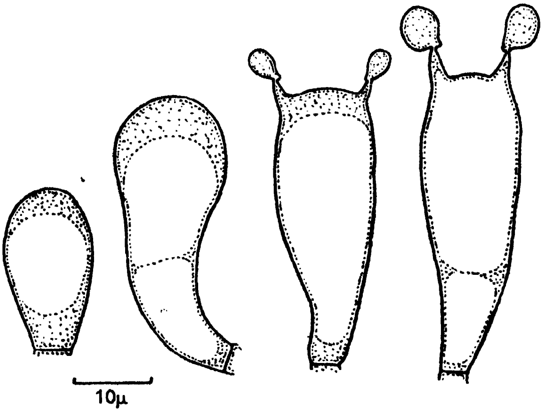

Fig. 6. Developing and mature (right) basidia of *Fomes subresinosus*, to show the absence of charging, × *c.* 1000.

That is, from the mean values of the basidium, one finds that the amount of protoplasm available for the spores corresponds closely with the volume of the four spores plus that of the sterigmata (which, in this case, do not become vacuolate).

In conclusion, it may be observed that the fruit bodies of this species grow remarkably quickly compared with those of *Amauroderma*, and such is to be expected if the protoplasm is not rapidly drained away from them by the discharge of normally grown spores. Nevertheless, the species is not uncommon, so that one must assume that the depauperate spores which are produced are capable of reproduction.

Hygrophorus sp. A common Malayan species of this genus, with small red fruit bodies resembling in shape those of *H. pratensis*, also produces unusually small spores on large basidia. Examination of the developing basidia shows in this case, however, that they become charged from below upward, instead of from above downward, and that the vacuolation of the full-grown basidium begins in the distal third (of greatest diameter) and, thus, discharges it mostly in the wrong direction (cf. Fig. 7). Indeed, the distal part never becomes charged and the hyaline cap which forms over this more or less vacuolate apex becomes itself finely vacuolate, and fills the rudimentary spores with minutely foamy cytoplasm. On extrusion of the sterigmata and spores, the small vacuoles near the apex of the basidium coalesce into one ellipsoid vacuole which gradually expands and fills the clavate apex. Only the hyaline, uncharged protoplasm distal to the main vacuole enters the spores which are shot off before the vacuole enters the sterigmata, but the main vacuole continues to enlarge and push the dense cytoplasm in the stalk of the basidium *back* into the subhymenial hypha. Thus the mature spores are small and hyaline, because they receive only the first part of the injection to which they are due, and this part is usually vacuolate, having become mixed up with the displaced vacuolation of the full-sized basidium, with the result that the spores, on being shed, soon dry out further; their contents shrink from the thin, but firm, wall and give the false appearance of an oil drop. The true charge of the basidium, on the other hand, is returned by the inverted mechanism to the subhymenium where, presumably, it is used in the production of new basidia. In this species, therefore, because of some derangement of the basidium, there is a continual postponement of the depletion of the hymenium: instead of progressive exhaustion as normally charged spores are shot off, the hymenium hesitates. The gills of the fruit body become unusually thick, and the fruit body itself, instead of lasting merely 5–12 days in the sporing state, as is normal for *Hygrophorus* in Malaya, commonly persists, when the weather is favourable, for 3 or 4 weeks. I have not seen another kind of spore produced by this widespread and abundant species, so that one must assume, as in the preceding case of *Fomes subresinosus*, that its depauperate spore is capable of reproduction. The normal spore of this *Hygrophorus*, as shown from the following calculations, should be *c.* 11·5 × 6 μ, which is the typical size for the genus.

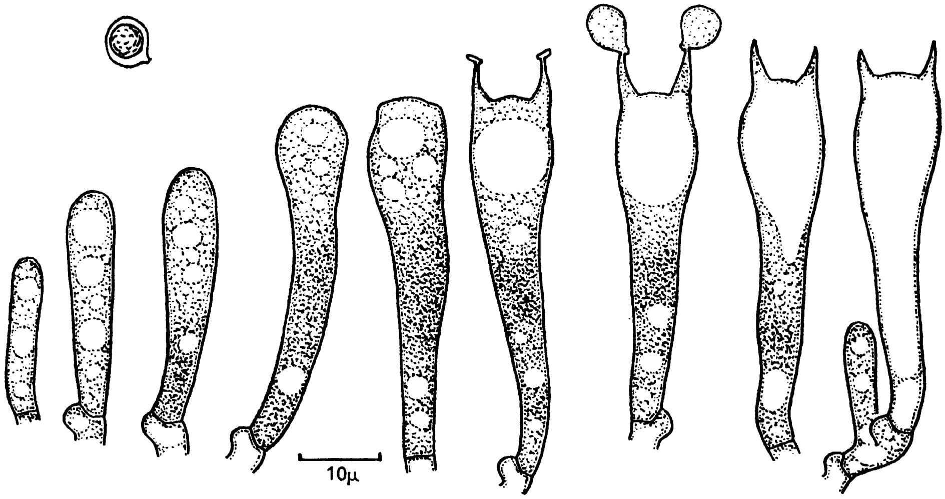

Fig. 7. Developing, mature and discharged basidia of *Hygrophorus* sp. (Malaya), to show how the basidium becomes charged in the proximal half and, when full sized, discharges by a subapical vacuole, first into the spores and then into the subhymenium; top left, a mature, air-dried spore with contracted contents. × *c.* 1000.

Mean size of basidium $= 51 \times 9{\cdot}5 \times 3{\cdot}5\,\mu = 1692$ c.μ (V).

Mean size of sterigma $= 6 \times 2{\cdot}5\,\mu = 9{\cdot}8$ c.μ (s).

Mean size of spore $= 5{\cdot}75 \times 5\,\mu = 75$ c.μ (v).

Mean size of the main vacuole in the apex of the basidium at the time of spore discharge $= 10{\cdot}5 \times 8{\cdot}5\,\mu = 397$ c.μ.

Also $$n\,(v+s) = 4\,(75 + 9{\cdot}8)\text{ c.}\mu = 339\text{ c.}\mu.$$

Thus, the volume of the four spores and sterigmata roughly equals that of the enlarged vacuole at the apex of the matured basidium (the enlargement of this vacuole displacing the apical protoplasm into the spores).

Now, assuming that $i = 2$, as in *H. firmus* and as seems likely from the appearance of the basidium, then if the basidium worked normally, the volume of the theoretical spore would be $$v = V \div in = 1692 + 8 = 211{\cdot}5\text{ c.}\mu.$$

Assume, also, that $w = 1{\cdot}6d$, where w is the width of the basidium and d that of the spore (as a general relation in *Clavaria, Omphalia, Hygrophorus* and other genera with similar basidia of this size). Then $$d = 9{\cdot}5\,\mu \div 1{\cdot}6 = 5{\cdot}94\,\mu.$$

Lastly, assuming the volume of the spore to be comparable with that of an ellipsoid of axes D and d then $$v = \pi D d^2/6,$$

whence $D = 11{\cdot}45\,\mu$, and the normal spore of this fungus should be *c.* $11{\cdot}5 \times 6\,\mu$.

Clavulina. Clavaria rugosa and its allies (cf. Table 1, nos. 38–49) have narrowly clavate, or subcylindric, basidia producing only two spores (rarely one) on rather strongly incurved sterigmata. The genus *Clavulina* was made by Schroeter in 1888 for this group of white-spored *Clavarias* because of the 2-spored character. Generally, in Homobasidiomycetes, this feature has no such value, being often merely varietal, and so Schroeter's genus has seldom been used. I find, however, that some twenty tropical species belong to the same group and that their basidia have other marked peculiarities of which the 2-spored character is an expression. It is not possible, yet, to appreciate the *Clavulina* basidium fully, but it belongs to the *Clavaria* kind (Corner, 1947) and has:

(i) never more than two spores;

(ii) $w = 0{\cdot}85d$; that is, the spores are wider than the basidium;

(iii) in most species, after spore discharge, one or more secondary (not clamped) septa form in the proximal part of the basidium;

(iv) the ratio $V : nv$ is about 2 (see Table 1, nos. 38–49), which suggests that the full-sized basidium is half filled with vacuole, whereas observation shows that the vacuoles occupy a much smaller portion.

The first and second features suggest affinity with the Dacryomycetales, but, as I will show in a later paper, the resemblance is superficial and the *Clavulina* basidium is really a modification of that typical of *Clavaria*.

Fig. 8 shows the basidia of a Malayan species of *Clavulina*. The basidium charges in the usual way from the apex downwards and for the greater part of its length, but the vacuole which discharges the dense protoplasm into the spores arises about one-third to one-half the length of the basidium from its apex. Thus, only the distal part of the basidium is emptied into the spores. After their discharge, a septum forms about the position where the discharging vacuole arose, and cuts off the depleted apex, which soon collapses, from the still turgid and but partly vacuolate base which proceeds to evacuate into the subhymenium, as in Fig. 6 for *Hygrophorus* sp. A second, third, and even fourth septum may form, each placed progressively nearer the base, exactly as Buller found in the evacuation of the mycelial hyphae of *Corticum Solani* (Buller, 1933, vol. 5, chap. 2, f. 74). The *Clavulina* basidium discharges in two directions, because of its central vacuole, as in *Hygrophorus* sp., but it develops normal spores because the direction of charging is normal. If the discharging vacuole were basal, it might be that four, instead of two, spores of nearly the same size as the dispores would be produced. However, the apex of the basidium is too narrow to hold a tetrad and it seems that the 2-spored character of the *Clavulina* basidium, as well as its other peculiarities, is related to its narrowness and inability to bear a tetrad: thus it resembles the *Dacryomyces* basidium in its hyphal form, but differs in more profound details of mechanism. The secondary septation of the *Clavulina* basidium is probably related with the presence of 4–6 residual haploid nuclei, for 6–8 are formed in the basidium and only one passes into each spore (Bauch, 1927). In *Hygrophorus* sp. there are probably no residual nuclei and, thus, no means of causing septation.

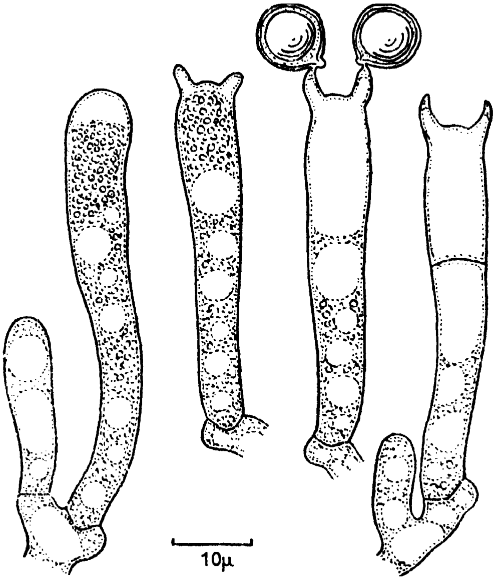

Fig. 8. Developing, mature and discharging basidia of *Clavulina* sp. (Malaya, aff. *C. rugosa*), to show how a central vacuole develops in the normally charged basidium to discharge first its distal part into the spores and, then, its proximal part into the subhymenium after the formation of a secondary septum. × *c.* 1000.

The discrepancy between *V* and *nv* in *Clavulina*, therefore, is not to be explained by the initial vacuolation of the basidium, but by its peculiar mechanism. The total-spore-volume must be related with the volume of the distal part of the basidium from which the spores are formed. I have, accordingly, studied three Malayan species in some detail, namely, *C. Léveillei*, *C. cartilaginea* and *Clavulina* sp. (= *C. gracilis mihi*, Fig. 8), and have found that the extreme and average positions of the secondary septum correspond with those of the distal end of the initial vacuole which discharges into the spores: the average position is *c.* 17 μ from the apex of the full-sized basidium. Table 10 will show that *nv* roughly equals, or closely corresponds with, the volume V_a of the basidium distal to the secondary septum. The approximation to unity in the last column of Table 10 shows that the distal part of the basidium must be filled with dense protoplasm which is transferred almost entirely into the spores.

THE AMPOULE EFFECT

It is convenient to have a name which will describe the normal working of the basidium. As a capsule filled to capacity, which empties itself on release, the basidium may be likened to an ampoule containing a certain dose. The basidium injects its dose into the spores and, as an ampoule is discarded after use, the basidium dies without means of recharging. The spores (or ampullulae) may, in their turn, inject their contents into sporidioles (or as Cicero might have said, ampulluliunculae). I propose to use, therefore, the expression 'ampoule effect' which was suggested to me by Dr R. Hill, F.R.S.: it has the advantage of directing attention to the basidium, as the mother-cell, rather than to the spores, which, as the free offspring, have hitherto been studied with most interest. The expression implies an effect dependent on a given quantity and it is the peculiarity of the basidium to prepare this quantity and to divide it equally among the spores by slowly squirting it into them.

Table 10. *Basidium size and spore size in* Clavulina

	Spore size (μ)	Basidium size (μ)	Septal distance (μ)	nv ($= 2v$) (c.μ)	V (c.μ)	V_d (c.μ)	$V : nv$	$V_d : nv$
C. sp. (Malaya):								
Maximum	9·5 × 9·5	65·0 × 9·0 × 6·0	24	898	2871	1268	3·2	1·4
Mean	8·5 × 8·5	53·0 × 8·0 × 5·0	18	644	1800	817	2·8	1·3
Minimum	7·5 × 7·5	40·0 × 7·0 × 4·0	12	442	980	398	2·2	0·9
C. cartilaginea:								
Maximum	10·0 × 8·5	50·0 × 8·0 × 6·5	20	758	2035	664	2·7	0·9
Mean	8·5 × 7·3	40·0 × 6·5 × 5·0	16	397	1050	397	2·2	1·0
Minimum	7·0 × 6·0	30·0 × 5·0 × 3·5	12	264	416	217	1·6	0·8

Septal distance = distance of secondary septum from apex of basidium.
V = volume of basidium, V_d = volume of basidium distal to the secondary septum.
v = volume of spore, n = number of spores per basidium.

The normal mechanism for the ampoule effect may be summarized in the following way:

(1) The developing basidium, started as a hyphal tip, charges and expands by filling up with dense protoplasm from the apex toward the base. Presumably, anabolites diffuse from the subhymenium into the basidium which, by condensing them into reserve substances, sets up and maintains the diffusion gradient, until the basidium is filled with dense protoplasm.

(2) A rearrangement of the contents of the basidium, on reaching full size, puts them in the following basipetal order:

(*a*) the hyaline cap of optically homogeneous material for the walls of the sterigmata and spores,

(*b*) the main body of dense protoplasm, further dehydrated by the extrusion of new vacuoles to form

(*c*) the basal and initial vacuolation of the full-sized basidium.

(3) The full-sized basidium discharges through n sterigmata and forms on each a spore of volume $1/n$th that of the dense protoplasm in the basidium. The motive force is

supplied by the osmotic pressure of the initial vacuole which draws water from the subhymenium and drives the distal end of the vacuole as a plunger toward the apex of the basidium, thus pushing the dense protoplasm into the spores. When the initial vacuolation begins, it seems that charging stops, and any protoplasm between the vacuole and the basal septum is unable to enter the spore. The sterigmata and spores are passively extruded and assume their shapes by distension and acropetal hardening of their walls against the internal pressure of the basidium.

(4) The spores are cut off before the vacuole of the basidium can enter them (possibly, thickening of the wall of the apiculus closes the sterigmatic canal). Dissolution of the walls of the sterigmatic necks then allows the successive ejection of the spores: first a drop of vacuolar sap is forced through the apicular side, as Buller's drop, to be followed in a few seconds by rupture of the neck and the emission of a minute jet of residual cytoplasm, or vacuolar sap, which propels the spore.

(5) If V is the volume of the basidium, A that of the initial vacuole, v that of the spore and n is the number of spores on the basidium, then

$$V - A = nv,$$

or

$$V = inv,$$

where $i = V/V - A$ and i is the index of initial vacuolation. Thus, in normal basidia, i can be taken as the ratio $V : nv$, and the practical difficulty of estimating the degree of vacuolation can be overcome. (The characteristic i seems to vary considerably among the species of a genus).

(6) Variations on the normal working of the basidium arise not from alternative mechanisms but from faults such as incomplete charging (giving a high value to i), or the displacement of the initial vacuolation to a central and, even, subapical position (thus preventing the protoplasm in the proximal part of the basidium from entering the spore).

It is to be hoped that it will be possible to make a model of the basidium, but it will be necessary to find a substance which, on extrusion, sets more or less rapidly at the surface to simulate the acropetal hardening of the hyphal wall, whether of hyphal tip, basidium apex, sterigma or spore. Realistic details, however, must wait analysis of the spore spacing at the apex of the basidium.

The method of staining the vacuole with neutral red (Chadefaud, 1937) should be employed along with the study of the living basidia: the first to resolve finer details of structure, and the second to observe truly the sequence of events. This author's statement that the water in the vacuole plays a purely passive role in the development of the basidium is mistaken, for the basidium is a water-vacuole, or hydrostatic, mechanism.

THE NOMENCLATURE OF THE BASIDIUM UNIT

As the spores are part of the basidium unit and are extruded symmetrically from the basidium, they can be described most readily by reference to the main, or long, axis of the basidium about which it is a figure of revolution. Thus, in Fig. 9, I have shown what seems to be the most useful and direct nomenclature of the parts, especially of the asymmetric basidiospores. *Apiculus* is the systematist's term for Buller's *hilum* (Buller, 1922, vol. 2, chap. 1): it is part of the base of the spore and is not a scar of detachment. The expression 'lateral apiculus' is erroneous, cf. Fig. 3, G, H. I have used 'abaxial' and 'adaxial' to describe the two sides of the spore in median longitudinal section because the terms in use are ambiguous and undesirable. It was customary to call the adaxial side the dorsal and the abaxial side ventral, which is the opposite of botanical usage for appendages of an organ, e.g. petals of a flower. Josserand (1937) has pointed out this confusion of thought and has added that the dorsal side of the basidiospore, though not the ventral, is commonly the ventricose. He proposed the terms 'l'arête interne' and 'l'arête externe' as unambiguous; but which is which? By the first, I understand the working face of the spore wall in contact with the cytoplasm, hence my use of adaxial and abaxial for the spore as a lateral, or cyclic, appendage of the basidium apex.

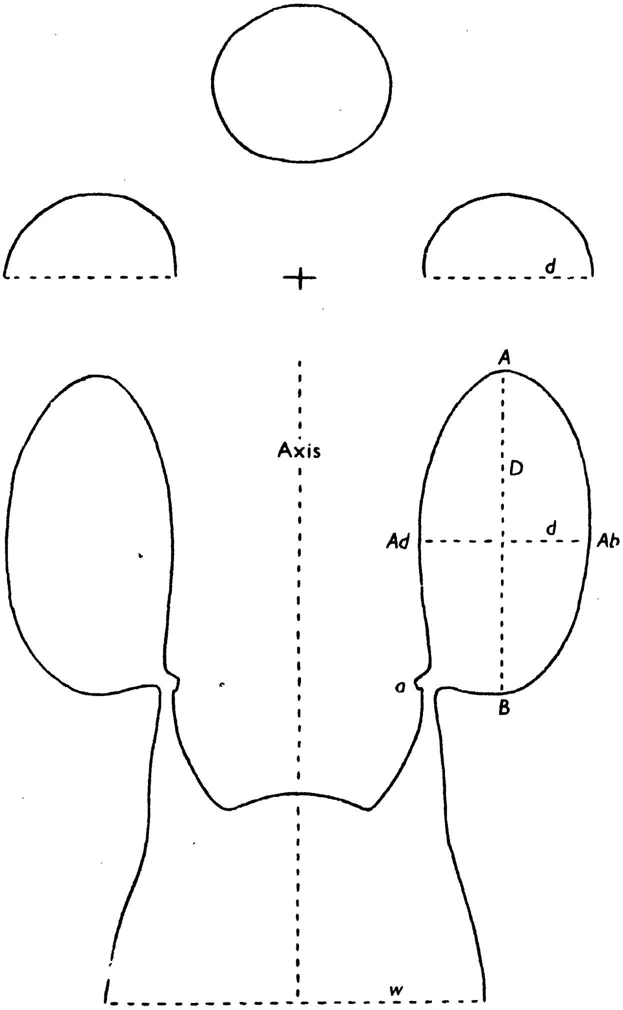

Fig. 9. A diagram of the structural relations at the apex of the basidium unit, as shown by a median longitudinal section of the mature basidium apex distal to its plane of greatest width (*w*) and by a terminal view of half the spore tetrad (above). *Axis*, the longitudinal axis of the basidium unit; *A*, the apex, and *B*, the base of the basidiospore; *Ad*, the adaxial side, and *Ab* the abaxial side of the basidiospore; *D*, the length, and *d*, the width of the basidiospore; *a*, the apiculus; *w*, the greatest diameter of the basidium.

SUMMARY

The development of basidium, as seen in its living state, is described by using *Oudemansiella* (*Collybia*) *Canarii* as a type. Seven stages are distinguished, namely (i) inception as a hyphal tip, (ii) charging with dense protoplasm, (iii) initial vacuolation of the full-sized basidium, (iv) development of the sterigmata, (v) development of the spores, (vi) discharge, and (vii) collapse. The main figures are the fully charged basidium, the full-sized basidium (as the fully charged extended by initial vacuolation) and the mature basidium with full-sized spores and fully vacuolate body.

A *hyaline cap* of homogeneous cytoplasm occupies the apex of the full-sized basidium as the antecedent to the sterigmata and spore initials. The part of the basidium apex from which a sterigma arises is called a *sterigmatic patch*.

The basidium is regarded as a self-charged ampoule containing dense protoplasm and basal vacuole (as plunger). Enlargement of the basal vacuole injects the dense protoplasm into the spores. If V is the volume of the basidium, A that of the initial vacuole, v that of a spore, and n is the spore number, then for practical purposes

$$V-A=nv,$$

and the ratio $V:nv$ is called the index of initial vacuolation of the full-sized basidium.

Proof of this relation is given (i) by comparison of basidium volume with total-spore-volume in species of *Clavaria*, *Oudemansiella*, *Amanita* and *Hygrophorus*; (ii) by comparison of spore number with spore volume in a wide range of Basidiomycetes.

Three exceptions (*Fomes subresinosus*, *Hygrophorus* sp. and *Clavulina*) show that apparent anomalies, leading to the production of small spores from large basidia or normal spores from disporous basidia, are caused not by a different basidium mechanism, but by disturbances in the normal *ampoule* effect.

The use of the terms *adaxial* and *abaxial* to describe the asymmetric basidiospore is explained.

REFERENCES

Bauch, R. (1927). Untersuchungen über zweisporige Hymenomyceten. II. *Arch. Protistenk.* **58**, 285–99.

Buller, A. H. R. (1922, 1924, 1933). *Researches on Fungi*, **2**, **3**, **5**. London.

Cayley, D. M. (1936). Spores and spore germination in wild and cultivated mushrooms. *Trans. Brit. Mycol. Soc.* **20**, 225–41.

Chadefaud, M. (1937). Le cytoplasme et le vacuome des basides. *Rev. Mycol.* **2**, 98–108.

Corner, E. J. H. (1934). An evolutionary study in agarics: *Collybia apalosarca* and the veils. *Trans. Brit. Mycol. Soc.* **19**, 39–88.

Corner, E. J. H. (1936). *Hygrophorus* with dimorphous basidiospores. *Trans. Brit. Mycol. Soc.* **20**, 157–84.

Corner, E. J. H. (1947). Variation in the size and shape of spores, basidia and cystidia in Basidiomycetes. *New Phyt.* **46**, 195.

de Bary, A. (1884). *Vergleichende Morphologie und Biologie der Pilse, Mycetozoen und Bacterien.* Leipzig.

Donk, M. A. (1933). Revision der niederländischen Homobasidiomycetae-Aphyllophoraceae. II. *Med. bot. Mus. RijKuniv. Utrecht*, no. 9.

Ingold, C. T. (1939). *Spore-discharge in Land-plants.* Oxford.

Josserand, M. (1930). *Ann. Soc. linn. Lyon.* **76**.

Josserand, M. (1937). Champignons de la région Lyonnaise. *Bull. Soc. mycol. Fr.* **53**, 176–8, 225, fig. 17.

Maire, R. (1926). Remarques sur les causes de divergence entre les auteurs au sujet des dimensions des spores. *Bull. Soc. mycol. Fr.* **42**, 43–50.

Sass, J. E. (1929). The cytological basis of homothallism and heterothallism in the Agaricaceae. *Amer. J. Bot.* **16**, 663–701.

Singer, R. (1945). New and interesting species of Basidiomycetes. *Mycologia*, **37**, 436–9.

Smith, A. H. (1934). Investigation on two-spored forms in the genus *Mycena*. *Mycologia*, **6**, 305–312.

THE LOSS OF CHROMATOPHORES IN *EUGLENA GRACILIS*

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(With 13 figures in the text)

CONTENTS

	PAGE
I. Introduction	53
(*a*) Colourless forms of *Euglena gracilis*	53
(*b*) The significance of Zumstein's and Ternetz's results	53
(*c*) Later observations	54
(*d*) Problems awaiting solution	55
II. The organism: *Euglena gracilis*	55
(*a*) Occurrence in nature	55
(*b*) Morphology	56
(*c*) Physiology	56
(*d*) The strains employed	57
III. The colourless strains of *Euglena gracilis*	59
(*a*) The M-strain	59
(*b*) The A-strain	60
(*c*) Comparison with Ternetz's results	61
(*d*) Comparison of green and colourless strains	63
(*e*) Experimental attempts to induce apoplastidy	64
IV. *Astasia longa* and its relation to *Euglena gracilis*	66
(*a*) Occurrence of *Astasia longa*	66
(*b*) Morphology of *Astasia longa*	66
(*c*) Physiology of *Astasia longa*	66
(*d*) Comparison of *Astasia longa* with the apochlorotic *Euglena gracilis*	68
V. Étude cytologique d'Euglènes vertes et incolores du type *Euglena gracilis*, par Raymond Hovasse	68
(*a*) Introduction et technique	68
(*b*) Les Euglènes incolores	70
(*c*) Les Euglènes vertes	73
(*d*) Chondriome et plastidome; la disparition momentanée de la chlorophylle	75
(*e*) Action du milieu de culture sur la disposition et l'importance du chondriome	78
VI. Discussion	79
(*a*) Cytological aspects	79
(*b*) Ecological aspects	80
(*c*) Taxonomic aspects	81
(*d*) The concept of leucophytes	84
VII. Summary	85
References	85

I. INTRODUCTION

(*a*) *Colourless forms of* Euglena gracilis

Euglena gracilis was found by Zumstein (1900) to multiply in the dark if suitable organic substances were available. Unlike other algae it contains no chlorophyll under these circumstances. Zumstein concluded that the difference between *Euglena* and *Astasia*, which is mainly based on the pigmentation of the former, loses much of its significance through this observation.

Klebs (1893) endeavoured to differentiate between the two genera on the basis that *Euglena* divides in a non-motile, *Astasia* in a motile state. This difference, although it holds good in most cases, has lost much of its value, since several species of *Euglena* are able to divide without shedding their flagella (Mainx, 1927, p. 317), while a few *Astasiae* can multiply in a palmelloid state. The relation between *Euglena* and *Astasia* is far from clear. The general opinion that *Astasia* is derived from green ancestors has not led to the comparison of individual species of the two genera. Since *Astasia* is now better known (Pringsheim, 1936, 1942) such comparisons should be possible, but they have not as yet afforded helpful clues.

Zumstein's results were confirmed by Ternetz (1912), who found in addition that under certain circumstances the cells of *Euglena gracilis* lose their chromatophores altogether, so that the progeny, the so-called hyaline light form ('hyaline Lichtform'), can never again become green as does the form grown in the dark ('hyaline Dunkelform'). The former has also lost the yellow accessory pigments present in the leucoplasts of the form grown in the dark. The hyaline light form discovered by Ternetz is therefore not only apochlorotic but apochromatic and, what is of special interest, it has also lost its eye-spot. For these various reasons Ternetz's form is not distinguishable from a true *Astasia*.

(*b*) *The significance of Zumstein's and Ternetz's results*

The fact, that *Euglena gracilis* can be grown on organic substances in the dark, and that its chromatophores are then reduced to small yellowish or almost invisible leucoplasts, which in the light become green again, is theoretically and experimentally of greater importance than is generally realized. The capacity for saprotrophic growth enables the apochromatic form of this species, in contrast to all the others so far tested, to survive the complete loss of chromatophores. The behaviour of the leucoplasts in the light provides convincing evidence that the form, which can no longer become green, is really apoplastic, i.e. devoid of any kind of plastids.

Ternetz's experiments were based on those of Zumstein and were performed with the same strain. Like the latter author she used peptone broth to which citric acid was added, and by repeatedly transferring cells to this strongly acid medium she obtained bacteria-free cultures (Ternetz, 1912, p. 443). Cells devoid of chromatophores occurred in small numbers among thousands of green ones, and the colourless form could be isolated and grown separately, although it did not multiply as vigorously as the green form (p. 503).

Ternetz favoured the view that the hyaline light form resulted from a lack of harmony between cell division and multiplication of chromatophores (p. 500). In very dilute acid media the number of the latter became considerably reduced so that, according to her, individuals with one single chromatophore were ultimately obtained. Under conditions inducing quick multiplication such individuals were stated to give origin to daughter cells with and without a chromatophore respectively.

This plausible explanation is, however, improbable on several grounds. (1) Ternetz (p. 475) describes an 'intermediate form' with yellowish chromatophores and a reduced stigma, which, by further multiplication, gives rise to green, yellowish and completely colourless cells, the last evidently identical with the hyaline light form. In the intermediate form the disappearance of chromatophores obviously takes place by degeneration, not by unequal distribution. Is it justifiable to assume that the hyaline light form can originate in two different ways? (2) The hyaline form is also devoid of an eye-spot and its disappearance is taken as a sure indication that regeneration of the chlorophyll apparatus has become impossible. How can the occurrence of an eye-spot be affected by a lack of harmony between the division of the chromatophores and of the whole cell?

This part of Ternetz's rightly much-valued paper conveys the impression that she gave preference to the disharmony theory owing to its elegance. She herself admits that apoplastic cells only occurred occasionally. This should not be so, if the sequence of events leading to individuals without chromatophores could be brought about at will. A more probable assumption is that the strain, either owing to hereditary constitution or as a result of previous influences, was in an unstable state, so that sudden change in conditions caused degeneration of the chromatophores and of the eye-spot. This would be in agreement with the view expressed by Beijerinck (1904, p. 235) in relation to the somewhat similar case provided by his *Chlorella variegata*.

The strain used by Zumstein and Ternetz has unfortunately been lost so that the question whether it could have been a variety with certain aberrant properties, distinguishing it from other strains of *Euglena gracilis*, cannot be directly answered. Frank (1904) employed this strain for experiments on irritability. When I attempted to repeat these with another strain (Pringsheim, 1908), I observed certain deviations in its behaviour and nutritional properties and concluded that it belonged to a physiologically different race (Pringsheim, 1912, p. 38). Lwoff (1932, p. 92), in view of its differing from other strains of *E. gracilis*, which do not give rise to colourless individuals, even called the Basel strain var. *Zumsteinii*.

(*c*) *Later observations*

Apochlorotic cells of this species have only once since been recorded, namely by Elmore-Sauer (1928, 1935). She carried out some interesting work with them but, as I learned from a personal letter in 1936, did not observe the actual loss of chromatophores. She maintained a dozen stock cultures continuously in the dark for 5 years and was surprised, when she attempted to study the conversion of the colourless into the normal green strain, that one of the cultures did not revert to the green form. Mrs Sauer was of the opinion that darkness offered favourable conditions for the survival of the hyaline form. That may well be so because they are then placed on an equal footing as regards competition with the green form. Her results, though confirming Ternetz's observations, do not add much to their explanation. Fortunately her strain (No. 2 of the list on p. 58) has been preserved in the green state.

All the several strains isolated by Mainx (1927) and myself in Prague never showed any tendency to become colourless. Only once, in 1937, at Lunz, Lower Austria, did

I find a race with partly degenerating chromatophores, recalling Ternetz's unstable intermediate form, although attempts to obtain a completely unpigmented substrain met with no success. C. L. Baker (1933) claims to have observed all three of Ternetz's forms, but he did not succeed in isolating an apochromatic strain (p. 436) which is not surprising in view of his primitive technique. Even if Bolte's (1920) record of the replacement of *Euglena gracilis* in the dark by the stable hyaline form is to be explained by the presence of an *Astasia* in the original mixed material, degeneration of chromatophores has definitely occurred repeatedly and at several places.

(*d*) *Problems awaiting solution*

A repetition of Ternetz's experiments seems desirable since improvements in technique afford better prospects of success in solving some questions that still remain open. Ternetz did not use media really suitable for a rich saprophytic growth, but since Dusi (cf. p. 56) has shown that *Euglena gracilis* is greatly favoured by acetates, it is easily grown in quantity. There is, moreover, some doubt whether Zumstein's and Ternetz's cultures were really bacteria-free, and certain irregularities in their results (Zumstein, 1900, p. 191; Ternetz, 1912, pp. 448, 483) may have been caused by bacteria.

Apart from questions arising in the course of my investigations, certain problems presented themselves at the outset, viz. (1) Could every strain of *E. gracilis* be induced to produce apochlorotic cells? (2) How does the disappearance of the chromatophores actually take place? (3) What physiological differences exist between green and apochlorotic strains? (4) Is it possible to obtain apochlorotic strains which have not lost their eye-spot? (5) Are other morphological and cytological changes connected with the loss of chromatophores and eye-spot? (6) What is the relation between the apochlorotic form of *E. gracilis* and species of *Astasia*?

In seeking for an answer to any one of these questions a knowledge of the characteristics of *Euglena gracilis* is of primary importance.

II. THE ORGANISM: *EUGLENA GRACILIS*

(*a*) *Occurrence in nature*

Euglena gracilis, though not rare, is not one of the common species like *E. viridis* Ehrbg., *E. agilis* Carter (syn. *E. pisciformis* Klebs), *E. anabaena* Mainx and *E. deses* Ehrbg. The density of its population in nature is also generally inferior to that of other species, such as *E. viridis* Ehrbg., *E. sanguinea* Ehrbg. and *E. sociabilis* Dang. *E. gracilis* is in fact usually found in small numbers among other pigmented flagellates and algae, an occurrence which is in agreement with its ability to lead an autotrophic existence.

When material is brought into the laboratory *E. gracilis* sometimes multiplies enormously, especially if decaying plant remains are present (Pringsheim, 1912, p. 1). This can take place even in the dark where it might easily be mistaken for an *Astasia* with an eye-spot, were it not for the fact that it soon becomes green when exposed to light. The species is sometimes found amid debris, associated with organisms characteristic of nutritive deficiency. It then exhibits signs of starvation, such as small size, absence of reserve substances and translucency, combined with slow movement and usually with a reduction in the dimensions and the pigmentation of the chromatophores and with appearance of reddish brown particles in the cells, so that the individuals are rather faintly coloured (Pringsheim, 1912, pp. 38 *et sqq.*). Individuals which can easily be mistaken for colourless ones are, on the other hand, found in rich but unbalanced media. Their cells are full of paramylon and the whole culture presents a whitish appearance. The cells found during the first days in beef extract media by Zumstein (1900, p. 185; cf. also Ternetz, 1912, p. 467) may have been of this kind. It is necessary to be careful not to confuse such seemingly colourless populations with truly apochlorotic strains.

(*b*) *Morphology*

As in other species of the genus, *Euglena gracilis* is rather variable, but it is not clear whether it 'includes several morphologically related species' (Lackey, 1940, p. 279), or whether environmental factors have a marked influence on its appearance. Although cultural experiments show that some of the differences are actually hereditary, they are scarcely sufficient to warrant describing the aberrant forms even as varieties. All the strains examined by other investigators and myself possess the following specific features: an elongate almost cylindrical shape, a moderate degree of metaboly, rounded or polyhedral chromatophores with pyrenoids provided with a double sheath of paramylon, a small eye-spot and a flagellum of almost the same length as the extended body. The nucleus is either central or situated behind the middle, never in front of it. In the living cell it shows no internal structure and appears as a clear vesicle. The aperture of the large gullet is somewhat towards one side. The flagellum can sometimes, without special preparation, be traced into the gullet, where there are two flagellar 'roots'. The surface striation of the cell is delicate. Palmella stages may be formed, but cysts with a distinct gelatinous envelope have not been observed. This description agrees with Klebs's (1883, p. 303) original diagnosis, as well as with the revised one given by Zumstein (1900, p. 160), which includes the hyaline dark form (cf. also Dangeard, 1901, p. 187).

(*c*) *Physiology*

Euglena gracilis can multiply either autotrophically in the light (Pringsheim, 1912; Mainx, 1927; Dusi, 1930; Hall, 1939*a*; Hall & Schoenborn, 1939), or heterotrophically in the dark, when provided with suitable organic substances, such as beef extract, yeast autolysate, peptones and various decoctions. Its development is much favoured by the addition of sodium acetate (Lwoff & Dusi, 1929, 1931; Dusi, 1933, p. 592; Lwoff, 1932, pp. 84 *et sqq.*), and it can also grow in the dark in an ammonium acetate medium containing the necessary inorganic salts (Schoenborn, 1942). All the strains investigated show these and the following features, including decolorization in the dark.

As Zumstein and Ternetz have shown, *E. gracilis* multiplies in very acid media, being more resistant to these than other members of its genus (Linsbauer, 1915; Kostir, 1921). Citric acid, present in Zumstein's medium, has no nutritional value (Pringsheim, 1912, pp. 33 *et sqq.*; Ternetz, 1912, p. 452), while Dusi (1933, p. 592) has shown that various peptones admit only of poor multiplication. The growth in Zumstein's medium, also used by Ternetz, is therefore never rich and fails altogether when peptone and citric acid are sterilized together. The use of beef extract in place of, or in addition to, peptone improves the growth considerably (Pringsheim, 1912, p. 33), but presence of acetate is necessary to afford optimum growth when CO_2 assimilation is excluded. It was therefore employed in all the experiments described in the following pages.

Reduction of chromatophores is frequently observed in material of *Euglenae* from natural habitats, not only in *Euglena gracilis* but also in *E. deses*, *E. geniculata*, *E. proxima*. In cultures a certain reduction can be induced by media favouring cell division but poor in nitrogen compounds, such as soil or peat extract (Pringsheim, 1912, pp. 40–41). The cells in such cultures exhibit a reduced chlorophyll content and small, yellowish chromatophores. Such features are also found in other flagellates, for instance in *Haematococcus* (Reichenow, 1909, p. 87) and in Chrysomonadinae from bog pools (Scherffel, 1911, p. 337). It is doubtful whether the effect of such media is in any way connected with the process of apochlorosis. Schmitz (1884, p. 111, footnote) and Mainx (1927, p. 394) were of the opinion that all the apochlorotic *Euglenae* of Klebs and other authors were such modifications produced by unbalanced or exhausted media. Although this is not quite correct, both Klebs (1883, pp. 290 *et sqq.*) and Hansgirg (1886–8, p. 172) may not always have distinguished between the two phenomena.

The faculty of *Euglena gracilis* to multiply in the dark is rare in the genus. Other species undergoing chromatophore degeneration do not survive because of starvation, but *E. gracilis* is able to lead a heterotrophic existence and can therefore multiply even when devoid of chlorophyll.

The pH range for growth of the species is very wide (Jahn, 1931; Alexander, 1931; Dusi, 1933, pp. 11 *et sqq.*). It can grow in media down to about pH 3, but does not show a preference for such acid media over neutral ones. It also multiplies in alkaline media up to pH 8 and more. A sharper definition of the pH boundaries is useless, since they depend on the composition of the medium. Moreover, the pH value changes with the metabolism of the cells and is also influenced in the alkaline region by absorption of CO_2. Mainx (1927, p. 361) found that *E. gracilis* grew best in media with a neutral reaction, but that it readily withstood considerable changes, and this has been confirmed by later authors and by my own experience.

Proteins seem to be more suitable, the more completely they are hydrolysed (Mainx, 1927, pp. 369–70), the best growth being observed after consecutive treatment with pepsin, trypsin and erepsin. *E. gracilis* is, however, able to liquefy gelatine, an indication of the secretion of proteolytic enzymes.

Maximum growth cannot apparently be achieved in simple media. The most favourable of all those I prepared and tested are the following: sodium acetate 0·1%; beef extract Difco 0·1%; bacto tryptone Difco 0·2%; yeast extract Difco 0·2%; and sodium acetate 0·1%; tryptone 0·1%; yeast extract 0·2%. In these media all the different strains grow abundantly, both in the light and in the dark, although not all of them to the same density. Growth also takes place on agar provided with the same nutritive substances. The optimum temperature is near 25° C.

(*d*) *The strains employed*

The problem how to achieve a reduction of the chromatophores attracted my attention long ago (Pringsheim, 1912, p. 38; 1921, p. 130). Mainx (1927, p. 394) failed to obtain colourless cells in spite of the fact that he tested several strains. Material was therefore collected from various localities in the hope of finding a strain which would behave like that of Zumstein and Ternetz. One of Mainx's strains, also used by me, as well as by Lwoff and Hall and their collaborators, is still available. In addition, strains were investigated which had either been sent to me or had been isolated by me.

List of green strains of *Euglena gracilis* used in the experiments

(1) Isolated by F. Mainx (1927, p. 331) from the large, lake-like fish-pond near Hirschberg in Northern Bohemia.

(2) Sent to my Prague laboratory by Mrs Elmore-Sauer in 1936 (cf. Elmore-Sauer, 1928, 1935).

(3) Received in 1936 as a pure culture on agar from Prof. W. Vischer, Botanisches Institut, Basel.

(4) Isolated from material sent me by Sister Monica Taylor, Notre Dame Training School, Glasgow, in December 1939. The original population occurred in water of pH 6·5 with detritus and was associated with *Rhabdomonas costata* and other flagellates.

(5) Isolated from plankton, including diatoms and other algae, from Esthwaite Water, Lake District, received from Dr G. E. Fogg, Botany School, Cambridge, in May 1940.

(6) Isolated from a suspension of mud with *Distigma proteus*, *Astasia ocellata*, *Euglenopsis vorax*, etc., received from Prof. T. M. Harris, University of Reading, in June 1940.

(7) Culture on agar, received from Mr J. B. Lackey, U.S. Public Health Service, Cincinnati, Ohio, in November 1941.

(8) Isolated from a suspension of detritus with *Peranema trichophorum*, *Paramecium bursaria* and small algae, received from Sister Monica Taylor, in December 1944.

(9) Isolated from material sent me by Mr T. G. Tomlinson, Dept. of Scientific and Industrial Research, Minworth, nr. Birmingham, in January 1945. This was a decaying mucus, containing fungal hyphae and *Sphaerotilus natans*, from the cooling tower of a sugar-beet factory.

(10) Isolated from a raw culture, containing ciliates and *Chlorella*, received from Prof. D. L. Mackinnon, Zoology Department, King's College, London, in February 1945.

(11) Isolated from a sample received from Dr S. P. Chu, Wray Castle, Windermere, in July 1945.

(12) Isolated from mud suspension with *Chlamydomonas* and other green flagellates, received from Prof. T. M. Harris, in April 1940.

(13) Isolated from mud from the bed of the River Granta at the Bathing Place near Newnham, Cambridge, in July 1946.

All these strains are very similar in appearance and behaviour. Each was easily isolated in pure culture with the help of the pipetting technique (Pringsheim, 1937, p. 639; 1946*a*, pp. 70 *et sqq.*). They do, however, exhibit certain minor differences. Strain 6 more commonly contains reddish brown particles than the others, although they are sometimes present in these too, especially when the medium is exhausted. Strain 4, which also differs in its somewhat larger eye-spot, surpasses all the others in its multiplication rate. Strains 4 and 7 alone produced colourless cells in my experiments, leading to an apochlorotic progeny, although strain 2 had produced such previously also. The majority of the strains, namely 2, 3, 5, 8, 9, 10, 11, 12 and 13 were identical with no. 1, as far as can be judged by present experience.

III. THE COLOURLESS STRAINS OF *EUGLENA GRACILIS*

(*a*) *The M-strain*

Mention has been made of a population of pale, partly yellowish cells found in the fish-pond near the Biological Station of Lunz (cf. p. 54) and recalling Ternetz's intermediate form. Similar cells were encountered in material sent to me by Sister Monica, from which strain 4 was derived. I am very grateful to her for providing this material. Among the flagellates it contained were cells which could be mistaken for an *Astasia*, when observed under low power, and others obviously belonging to an *Euglena* and not quite as slender as the former. Both were negatively phototactic and otherwise similar in appearance. More careful examination under high power showed that the pale-coloured cells had a green tint and a small eye-spot, while the green individuals had the structure characteristic of *E. gracilis*, though with small reddish granules in the cells. A few specimens were intermediate between the two forms so that the latter were suspected of belonging to the same species and of being derived from the same ancestry (Fig. 1).

Single individuals were repeatedly washed to remove bacteria and transferred to various media. Multiplication was observed after 3 weeks and was vigorous after 6 weeks, especially in media with beef extract or peptone and sodium acetate. It was difficult to judge of the hue of the organisms owing to the large amount of paramylon deposited in the cells in certain media. This gives them a whitish appearance and may conceal the chromatophores to such an extent that the cultures scarcely show any green tint (cf. p. 65).

Among the single-cell subcultures colourless cells were most prominent in a medium with 0·2% beef extract and peat decoction, although even here most of the cells were normal. Colourless cells were found in smaller numbers in most of the media. Alone in peat decoction with 0·1% ammonium chloride, but without additional organic matter, all the cells of the scanty population were of the normal green type.

In the beef extract-peat decoction culture the cells did not contain much paramylon. Otherwise the population showed a variety of types. A few individuals possessed only a few chromatophores; others had a hyaline appearance and contained red granules, indicating a state of malnutrition, although they were active and of the normal shape and size. This was the condition in winter 6 weeks after inoculation. A week later single cells were again isolated, pale ones being selected as far as possible. Their recognition under the dissecting microscope was not altogether certain, although the colourless cells were now definitely smaller and more slender than the green ones, by contrast with their condition a week earlier, in a similar way as in the original material. All of the cells isolated developed into subclones, of which four were green and four colourless, although the latter also turned green later. At this stage the strain behaved like Ternetz's intermediate form, being composed of divers individuals. The colourless cells were possessed of eye-spots, a sure indication that they were still not definitely apochlorotic.

Isolation of single cells was undertaken again at a time when the pale-coloured cells began to show a starved appearance. A complete medium containing 0·2% sodium acetate, 0·2% peptone Vaillant and 0·2% beef extract was used. All eight cultures developed, of which three were completely colourless and remained so. The eye-spots had disappeared with the chromatophores. The sequence of events in cells with degenerating chromatophores was the following: (*a*) Some of the chromatophores turn yellow and break up into pieces. Red granules appear. (*b*) No green chromatophores are left, only yellowish remnants and minute reddish spheres. (*c*) The shape and paramylon content of the individuals are unchanged but no trace of pigments is left. (*d*) The cells are still motile, somewhat elongate and almost cylindrical, and more slender than the green ones. (*e*) The colourless cells contain less paramylon than the green ones. (*f*) Vacuoles appear,* the cells having diminished in size without altering their shape. Eye-spots normal, cells still phototactic. (*g*) Cells have ceased to move. They show metabolic deformation and no eye-spot. (*h*) Cells disintegrate. Degeneration is of course partly due to starvation caused by loss of chromatophores.

In order to provide a more complete picture and to facilitate comparison with, and explanation of, Ternetz's experiments, another series of cultures that afforded apochlorotic substrains will be described. Like two other colourless clones this was derived from the first clone, isolated in December 1939; but almost 2 months later. While most of the cells were of the normal type, quite a number appeared colourless. These no longer showed flagellar movement like their green associates, but occupied the bottom of the culture vessel, where they exhibited lively metaboly and presented a rather moribund appearance. Some of them contained disintegrating chromatophores. The result of the experiment was slightly different from that recorded above.

Single unhealthy cells, transferred to the complete medium, showed no multiplication after 3 weeks, although, judging by previous experience, it should have taken place. However, after 4 weeks, growth had occurred in three out of the four cultures, all the resulting cells being colourless, but some of them still possessing eye-spots. Subcultures on agar and in the liquid medium afforded individuals which were completely colourless and without eye-spots. The sediment that formed after some time was pure white.

These results were confirmed by further experiments. As a general rule the intermediate form gives rise to mixed populations. Cells with the most advanced stage of chromatophore degeneration multiply to form apparently white populations which, however, become greenish after some time, and then turn completely green. If colourless but stigma-bearing cells from a young culture are isolated, the subclones have the same appearance and fate. After the organic food in the medium is exhausted, the colourless cells starve, while those with green chromatophores still multiply. If the former are transferred at the right time, they may recover, although they are no longer able to form green chromatophores. Those still containing chlorophyll, even those with the slightest green tint, have an advantage in respect of multiplication in the light, especially if the organic food supply is inadequate, but in the dark colourless and normal cells develop at much the same rate.

(*b*) *The A-strain*

I am much indebted to Mr J. B. Lackey for sending me a strain (No. 7) from America under the name *Euglena agilis*† which proved very interesting. Originally a pure culture, it was contaminated *en route* with *Penicillium*. A little of the growth was transferred to a putrefaction tube, containing a wheat-grain, calcium carbonate and soil, to obtain plentiful material. After 2 weeks the strain was identified as typical *Euglena gracilis*. A week later cells were washed and inoculated into various media. The culture in acetate, beef extract and peptone Vaillant showed intense multiplication after 5 weeks and looked colourless but had still the eye-spot. Three weeks later, most of the cells were completely apochlorotic and had no eye-spot, while a few were green, of larger size and possessed of an eye-spot.

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* Vacuolization, accompanying the breaking up of the chromatophores, was already observed by Ternetz (1913, p. 476). It is a transitional stage in the transformation of the cell contents, the considerable space within the cell occupied by the chromatophores becoming replaced by the chondriome which increases in volume (cf. p. 75).

† The name *Euglena agilis* (Carter, 1856) should be reserved for the species later called *E. pisciformis* by Klebs (1883), since the two pyrenoids and the general shape of the body are recognizable in Carter's drawing. The confusion with *E. gracilis* dates from the time of Baker (1926) whose error was repeated by Hall & Jahn (1929), who treat *E. gracilis* and a form called *E. agilis* as different species although, judging by the figures, they are identical. Mainx (1927, p. 313) and Fritsch (1935, p. 731), from the facts known to them, rightly draw the conclusion that the *E. agilis* of Baker is identical with *E. gracilis*.

After fixation with Schaudinn's fluid measurements were made of well-preserved and extended individuals. Otherwise a random selection was made.

Colourless cells	Length (μ)	60	51	52	57	57	51	52	57	50	57
	Width (μ)	8	8	10	8	7	8	10	10	9	8
Green cells	Length (μ)	80	63	72	79	72					
	Width (μ)	14	12	14	11	14					

The differences in size are unmistakable.

Single apparently colourless cells were transferred to various media. All six cultures developed, four of them being completely colourless, the others green. From one of the colourless subclones four cells were individually transferred to new culture tubes. All the four cultures afforded apochlorotic substrains. Apart from the colourless clones green ones were also maintained.

No clue to the inherent cause of chromatophore degeneration was found by microscopical investigation. The disappearance of the green pigments was followed by that of the yellow ones and was accompanied by the deformation and diminution in size and number of the plastids (Fig. 1), which eventually disintegrated one after the other. These changes suggested the action of a parasite, no trace of which could, however, be detected. The healthy appearance and multiplication of the populations raised from apochlorotic cells speaks against parasitic attack being the cause of apochlorosis.

(*c*) *Comparison with Ternetz's results*

If a comparison is made only between the actual results without reference to their interpretation, Ternetz's and my findings are in good agreement. This can be illustrated by a series of quotations.

The intermediate form multiplies more rapidly than the hyaline form in the light (Ternetz, 1912, p. 479). The real cause of apochlorosis must be sought in an inner disposition rather than in external influences. After transference of cells from a dilute to a rich medium only occasionally cells exhibit loss of the capacity to form chlorophyll (p. 479), although such a change in environment is believed to initiate the loss of pigment. In cultures in the light there occur here and there, generally quite sporadically among thousands of green cells, single colourless individuals which disappear again after a few days (p. 481). The hyaline light form was observed only in thirty-five out of several hundred cultures.

Several of these statements are not in harmony with her theoretical explanation. If the transformation of green into apochlorotic cells were due to a certain constellation of external influences, such irregularities would not be expected. The observations seem on the contrary to show that the whole strain was genotypically of the nature of an 'intermediate form', although phenotypically it showed its true character only under certain conditions.

Ternetz has tentatively attempted to explain the genesis of the intermediate form by comparing the chromatophores of *E. gracilis* to the plastids in variegated leaves (p. 495), so that the permanent lack of pigmentation becomes due to the transformation of chloroplasts into leucoplasts. This explanation was abandoned because intermediate forms were so rarely observed. The incomplete character of Ternetz's observations prevented her therefore from grasping the real nature of the intermediate form which she seems to have regarded almost as constant, while in fact the intermediate cells change either into green or into apochlorotic ones and therefore disappear after a time. Ternetz (p. 496) does not consider the possibility that the transitional stages could have escaped her notice, and is of the opinion that such an assumption is superfluous, since she regards the phenomenon of apochlorosis as due to lack of harmony between cell and chromatophore division (Ternetz, 1912, p. 499; cf. also p. 53).

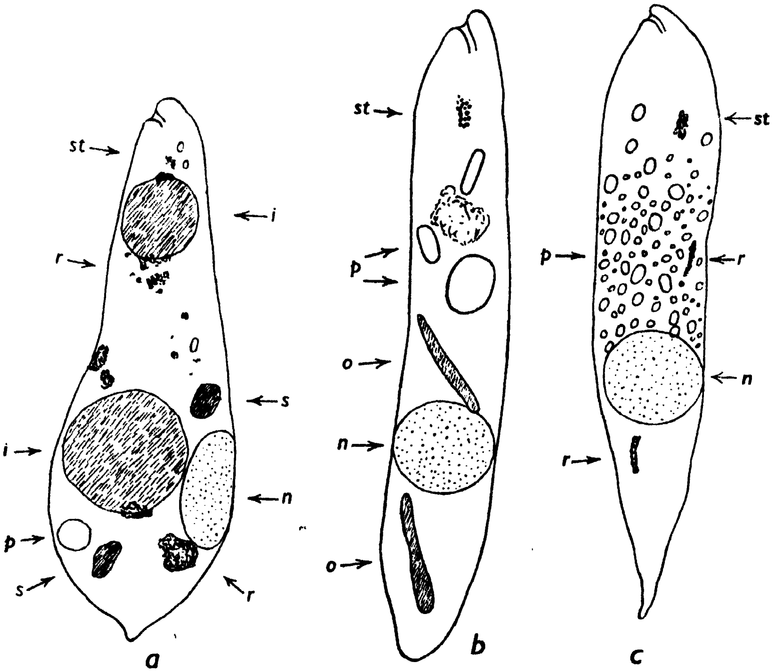

Fig. 1. Various stages of chromatophore degeneration in *Euglena gracilis*. × 1500. (*a*) Chromatophores in part inflated, in part showing subsequent shrinking; reddish brown particles in aggregates, little paramylon. (*b*) Chromatophores changed into orange-brown rods; shapeless masses in the cytoplasm, a few large paramylon grains. (*c*) Only few remains of plastids left; a considerable number of small paramylon grains. *i*, Inflated, and *s*, shrunken plastids; *r*, reddish particles; *n*, nucleus; *p*, paramylon; *st*, stigma; *o*, degenerate orange plastids.

Her own explanation is that the actual genesis of colourless cells takes place after transference from a very dilute medium, in which a reduction in the number of chromatophores was observed (p. 501), to a richer medium with an increased division rate. It is left vague whether the actual reason for the formation of apochlorotic cells is the reduction in the number of plastids or the speeding up of cell division. If the number of chromatophores is reduced by lack of harmony due to malnutrition, then this factor should suffice. If, on the other hand, it is the rich medium which causes the ratio between cell and chromatophore division to be shifted in favour of the former, then this alone should lead to the origin of colourless cells.

In another passage Ternetz arrived at the conclusion that internal reasons are mainly responsible for the appearance of colourless cells. She remarks (p. 501): 'It is not possible to explain why colourless forms appear only in certain cases....The predominant influence of an inner disposition cannot be overlooked, since of the 35 cultures with hyaline cells not less than 16 descended...from the same original green material. We are therefore obliged to assume two different modes of genesis of the hyaline form which, however, lead to the same end, to apoplastidy.' (Translated.)

(*d*) *Comparison of green and colourless strains*

Green and apoplastidic cells differ only by the presence or absence of chromatophores and stigma (Fig. 2). With the disappearance of the last traces of plastids the ability to become green again has been lost. This observation supports the Schimper-Meyer theory according to which plastids originate only by division of plastids. This theory has been challenged by those who claim that chondriosomes, formed in the cytoplasm, can give rise to plastids (Guilliermond, 1934, p. 48).

If a normal strain of *Euglena gracilis* is to be compared with an apochlorotic one, the experiments must be carried out in the dark. Even small remnants of chromatophores favour multiplication in the light and render the intermediate form superior to the apochlorotic one, as Ternetz (1912, p. 479) already observed. In darkness, however, there is no difference in the multiplication rate of the green, intermediate and apochlorotic strains, when the same number of cells is inoculated into a suitable medium. The intermediate form, with its disintegrating chromatophores, its red granules and its occasional vacuolate cytoplasm, makes a pathological impression, while the green, the etiolated and the apochlorotic forms present a 'normal' healthy appearance, when properly nourished. This difference does not, however, affect the rate of division. Ternetz's observation (p. 504), repeatedly emphasized by later writers, that the apochlorotic form is scarcely able to maintain itself, must have been due to the use of an unfavourable medium. There is also no difference between the strains with respect to the amount of paramylon stored, whether this is formed from protein derivatives or from acetate.

The various green strains show certain differences in the multiplication rate, strain 7, and to a lesser degree strain 12, multiplying more rapidly than the others. This difference disappears in the dark, as well as in the apochlorotic substrain A derived from strain 7, when compared with other apochlorotic strains. It is apparently due to a better development of the plastidome in strains 7 and 12 than in the other strains.

The longevity of the cultures is likewise independent of the kind of strain and of the presence or absence of plastids, provided the cultures are kept in the dark and subjected to the same conditions. Ternetz (p. 474) found that cultures in the dark were short lived, but I have pointed out that motility persists longer in the dark than in the light (Pringsheim, 1912, p. 30). This observation was repeatedly confirmed subsequently, so that Ternetz's contrary experience must have been due to contamination, as is indicated especially by her reference to a putrid odour (pp. 474, 483). The long continued motility in the dark is due to a decreased tendency to produce palmelloid stages. Under certain conditions, for instance on agar, after the surface had dried out, all the strains, green and colourless, in the light and in the dark, showed the ability to multiply in a non-motile state. As long as the agar is covered with a layer of moisture, however, the cells can creep over the surface, so that daughter colonies can appear at a distance from the point of inoculation (Pringsheim, 1912, pl. 1, fig. 2). The cells can also move within a very soft agar medium (0·3–0·5% agar).

While in all these respects there is no difference between green and apochlorotic strains, they differ in the reaction to light. The green strains, whether grown in the light or in the dark, are markedly phototactic, whereas the apochlorotic races, which have lost the eyespot, no longer react in this way.

(*e*) *Experimental attempts to induce apoplastidy*

Only in three (Nos. 2, 4 and 7) strains was the occurrence of colourless cells observed, and in each of these only once. With respect to the Basel strain the situation is not clear. While Ternetz obtained apochlorotic substrains with the plastidome altogether absent, Zumstein's observation of colourless cells is only mentioned by Ternetz (p. 482), so that their actual nature remains doubtful, and true apochlorotic cells may have arisen only once also in this strain.

While Zumstein believed (Ternetz, 1912, p. 482) that the appearance of colourless cells was due to the addition of an acid or to the influence of certain salts, Ternetz claims to have found the conditions necessary for the production of hyaline cells. Was she really able to repeat the experiment at will?

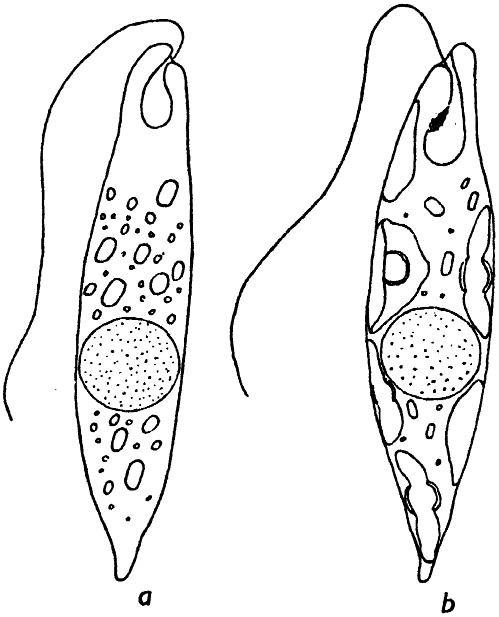

Fig. 2. Colourless (*a*) and green (*b*) cells of *Euglena gracilis*. × 1500.

I was unsuccessful in reproducing the phenomenon of chromatophore degeneration by artificial means in any strain, even in those which had previously given origin to apochlorotic substrains. The following experiments were carried out repeatedly and with variations, but without ever achieving a positive result:

(1) Cultivation in media with a low nutritional value. Such experiments seemed to offer some prospects of success because Ternetz (p. 500) has found the number of chloroplasts to diminish in dilute media, and because strain 4, which showed signs of plastid degeneration, arrived in a state of starvation, the scarcity of food being also indicated by the nature and appearance of the other organisms.

The nutrient solutions for these experiments were made in various ways, by diluting suitable media, by allowing decoctions of peas, hay or dry fruit to undergo microbial disintegration and by boiling peat with water. Cells with a subnormal number of chromatophores were often obtained, but degeneration or complete loss of plastids was never observed.

(2) Transference from poor to rich media was the means regarded by Ternetz (pp. 500–1) as effective for the production of colourless cells. One of my experiments, in which cells of strain 4 were transferred from peat extract to a complete and rich medium (cf. pp. 59, 60), was apparently successful, but further repetitions of this experiment failed.

(3) Transference of cells from a rich culture, either pure or with bacteria, to very dilute media, likewise failed.

(4) In a private letter Miss Sauer expressed the opinion that prolonged cultivation in the dark would increase the chances of obtaining colourless cells, but I have since established that repeated subculturing in the dark over a period of a year has no influence, nor has continued darkening of the same culture. Soon after exposure to the light the cultures became green again. Even when a culture was kept in the dark until it began to die, and a few cells, when isolated in a fresh medium, did not develop chlorophyll immediately, such cells did not give rise to colourless substrains. Experiments were carried out with cultures on agar, in liquid and in biphasic (Pringsheim, 1946*b*) media.

(5) Ternetz (p. 467) found colourless cells in a relatively concentrated beef extract medium during the first days after inoculation. I have been unable to confirm this observation, which seems to have been due, not to the special influence of the medium, but to the use of unstable cell material or to mistaking cells filled with paramylon as apochlorotic (cf. p. 69).

(6) According to Alexander (1931, p. 183), acid solutions near to the lethal pH affect *Euglena gracilis* in such a way that almost, though not permanently, colourless cells appear. Neither hydrochloric nor acetic acid produced such an effect in my experiments.

The means employed in the remaining experiments aimed at producing a specific effect on the cell contents and were suggested by Klebs's observation (1883, pp. 268–9, 292) that the chromatophores are the most sensitive organs of the cell, liable to be affected before the cytoplasm and the nucleus.

(7) Media with a low nitrogen content were made of peat extract with the addition of acetic acid or of acetate, in order to shift the ratio between the N-compounds and the N-free nutrients in favour of the latter. Growth in such media did not affect the plastidome.

(8) Ammonia was used because of its ready penetration into living cells, and because Euglenineae are known to live in habitats rich in this substance. Cells grown in the highest non-lethal concentration neither showed plastid degeneration nor did they give origin to other than green substrains, although the experiments were varied in several ways.

(9) An attempt was made to eliminate calcium from the medium with the object of influencing the state of the chromatophores, although *E. gracilis* (Pringsheim, 1926, p. 562) had been shown not to need calcium. Neither the use of the purest salts obtainable nor the addition of oxalate had any effect on the chromatophores.

(10) Since iron is indispensable for the formation of chlorophyll in higher plants and Emerson (1929*a*, *b*) has shown that *Chlorella* also does not produce chlorophyll unless there is a certain amount of iron in the medium, it seemed advisable to grow *Euglena gracilis* in media with as low an iron concentration as possible.

Removal of iron was accomplished in various ways, but neither autoclaving with $CaCO_3$ or $MgCO_3$ and subsequent decanting, nor the addition of tannic acid, if not too concentrated, had any influence on the growth and chlorophyll formation of *E. gracilis*. Decoctions of leaves containing tannic substances (*Quercus*, *Populus*, *Thea*) proved to afford quite good media, without producing any reduction in chlorophyll content or other effect on the chromatophores. The addition of 5 amino-acridine chlorhydrate, which is a very sensitive reagent for ferrous salts with which it forms red compounds, also had no effect.

(11) Other influences, such as heat and cold, treatment with penicillin and strong light were tested without better results.

The degeneration of the chromatophores in *Euglena gracilis* does not therefore seem to be due to external influences, which might be more detrimental to the plastids than to the remaining cell contents, but to internal influences exerted by the cytoplasm or nucleus, which I was unable to call into play.

IV. *ASTASIA LONGA* AND ITS RELATION TO *EUGLENA GRACILIS*

(*a*) *Occurrence of* Astasia longa

Astasia longa Pringsh. (Pringsheim, 1936, p. 66) was first found in two localities which were boggy. The more recently found strains, for instance the five enumerated by Pringsheim (1942, p. 189), came from similar habitats. In none of these was *A. longa* found in company with green *Euglenae*, although often associated with other Astasiaceae, such as *Menoidium pellucidum* and *Hyalophacus ocellata* (Pringsheim, 1936, p. 74). Of the strains still in my possession and all of them identical, one, which was brought to me by Dr S. P. Chu, was found in a bog near Wray Castle, Westmorland; there were again no green Euglenaceae present. The only aberrant form is *Astasia longa* var. *truncata* (Pringsheim, 1942, p. 190), which lacks the posterior apiculus and is shorter and more rounded at the back end. In its nutritional requirements and mode of growth on agar this strain is identical with *A. longa* typ.

(*b*) *Morphology of* Astasia longa

This species (Pringsheim, 1936, p. 66; 1942, p. 190) is elongate, almost cylindrical, with slight metaboly involving shortening and broadening of the body. The periplast is delicately striated, the reservoir at some distance from the front end, the gullet one-sided, the posterior end provided with a teat-shaped process; flagellum two-thirds body length; paramylon grains small, generally numerous, often lacking in front; nucleus central or behind the middle; palmellae observed. Pure cultures are easily obtained. These characters, which are common to all the strains, reveal a striking resemblance to *Euglena gracilis*, the only difference being the absence of chloroplasts and eye-spot (Fig. 3).

(*c*) *Physiology of* Astasia longa

Although this similarity was manifest from the outset, I was not prepared to accept the identity of the apoplastidic *Euglena gracilis* with *Astasia longa* without prolonged comparison of their physiological behaviour. Schoenborn's (1940, 1942) results with an *Astasia* isolated by T. L. Jahn helped to strengthen my conviction. An unidentified strain of *Astasia*, probably the same as Schoenborn's, sent to me by R. P. Hall, was found to be identical with *A. longa*. Schoenborn's *Euglena gracilis*, which was sent to me by Hall, was strain 4 of the list on p. 68. Although Schoenborn's results showed that the physiological behaviour of both forms was the same, he did not draw this conclusion nor did he regard *Astasia longa* as an apochlorotic race of *Euglena gracilis.*

Astasia longa grows well in 0·2–1% solutions of beef extract whether the reaction be slightly alkaline, neutral or strongly acid. Its multiplication is favoured by yeast extract, bacto tryptone and especially by acetate, and it multiplies on agar. It develops, though not abundantly, in Zumstein's Witte peptone-citric acid medium, provided both ingredients are sterilized separately. It can also multiply in a medium containing ammonium acetate and the indispensable accessory salts, but, in confirmation of Schoenborn's conclusion, it requires some accustoming to inorganic nitrogen, when transferred from a medium with peptone. In all these respects the behaviour of *A. longa* is identical with that of *Euglena gracilis*, when the latter is grown in the dark (cf. also Provasoli 1937–8, p. 106). *Astasia longa* is the only species of *Astasia* without an eye-spot and the only member of

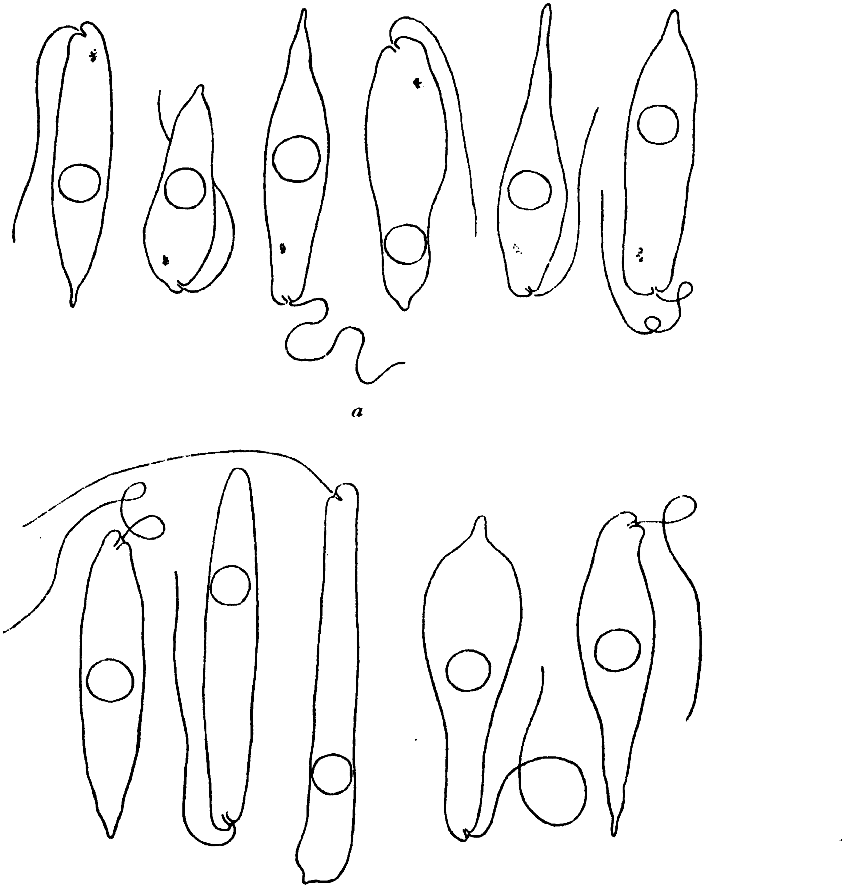

Fig. 3. Cells of *Euglena gracilis* (*a*) and *Astasia longa* (*b*). × 750. In spite of metabolic changes the shape and size are essentially the same.

its family that has been obtained in pure culture. Every effort has so far failed to isolate other species, although they are undoubtedly saprotrophic. The strains of *Astasia* sent to me by Lwoff and by Hall are also *A. longa*.

(*d*) *Comparison of* Astasia longa *with the apochlorotic* Euglena gracilis

The similarity between *Astasia longa* and *Euglena gracilis* becomes one of complete identity when the apochlorotic strains of the latter are taken for comparison.

The following features were specially investigated for this purpose: body shape, striation, size, flagellum, metaboly, formation of palmellae, flagellar movement; multiplication in various media, viz. with and without peptone and acetate, with ammonium as a source of nitrogen, in Zumstein's medium, in optimal media containing combinations of acetate, beef and yeast extract and tryptone; and growth on agar and gelatine. In none of the tests could any difference be detected. There can therefore no longer be any doubt that *Astasia longa* is but the apochlorotic race of *Euglena gracilis*. Ternetz's (1912, p. 437) conclusion that only the green form would be found in nature was therefore mistaken.

List of colourless strains of *Euglena gracilis* (syn. *Astasia longa*)

(1) Sent to my Prague laboratory as *Astasia chattoni* by A. Lwoff, Institut Pasteur, Paris (Pringsheim, 1942, p. 189).
(2) *A. longa* var. *truncata* (Pringsheim, 1942, p. 191).
(3) From Dr S. P. Chu, who found the material in September 1941 in a bog near Wray Castle, Lake Windermere where, prior to heavy rain, he had observed green *Euglena gracilis*.
(4) Isolated by T. L. Jahn and sent to me by Prof. R. P. Hall, New York, in June 1943 as a pure culture.
(5) Strain M derived from *E. gracilis* No. 4 (cf. p. 59).
(6) Strain A derived from *E. gracilis* No. 7 (cf. p. 60).
(7) From the boggy shores of a small lake near Garmisch, Upper Bavaria (Pringsheim, 1936, p. 67).
(8) From a ditch between Sphagnum swamps near Hirschberg in Bohemia (Pringsheim, 1936, p. 67).
(9) From a ditch at Cherry Hinton near Cambridge, September 1942.

(1)–(6) are those which were mostly used during these investigations; (7)–(9), which were isolated earlier, are essentially identical.

V. ÉTUDE CYTOLOGIQUE D'EUGLÈNES VERTES ET INCOLORES, DU TYPE *EUGLENA GRACILIS*

PAR RAYMOND HOVASSE

(*a*) *Introduction et technique*

Il subsiste encore, dans la cytologie des Euglènes, quelques points incertains, malgré les nombreux travaux qu'elles ont suscités.

Avant 1937, en effet, les recherches accomplies sur ces formes ont été le plus souvent, basées uniquement sur la méthode des frottis, qui, probablement par suite de l'existence de la cuticule, fournit des préparations difficilement utilisables pour une étude précise du

cytoplasme. Aussi, les données de D. Causey (1926), V. E. Brown (1930), C. L. Baker (1933), M. Chadefaud (1937, 1944), sont-elles le plus souvent incomplètes. Elles ont parfois même conduit à des interprétations inexactes.

Dès 1937, à propos des Volvocales, j'ai fait remarquer la nécessité d'étudier sur coupes minces les formes à membrane épaisse. C'est en partie ainsi qu'ont été éxécutés les travaux de A. Hollande (1938, 1942), auxquels nous devons les résultats les plus modernes concernant les Euglènes.

Toutefois, sa technique est encore imparfaite, probablement faute d'un emploi courant de la méthode d'inclusions mixtes à l'agar-paraffine de E. Chatton. Elle ne lui a permis de reconnaître avec certitude que le Golgi. S'il a décrit le chondriome des formes incolores, il n'a pu mettre en évidence celui des formes vertes; il ne s'est pas, d'autre part préoccupé des relations possibles du chondriome avec les plastes. Seul, Baker a jusqu'alors abordé ce problème, qu'il n'a pu résoudre, faute d'avoir reconnu le chondriome.

Grâce aux cultures de E. G. Pringsheim, dont j'ai pu disposer très largement, j'ai repris ces questions par l'examen d'Euglènes du type *Euglena gracilis* vertes et incolores. E. G. Pringsheim m'a fait bénéficier de sa grande expérience de ces formes, et a ainsi considérablement facilité ma tâche: je l'en remercie très cordialement.

Ma technique dérive de celle que j'ai mise au point pour les Volvocales depuis 1936: elle consiste à fixer le plus rapidement possible. Par l'acide osmique en solution à 2%, la fixation ne doit durer que quelques secondes, sinon les colorations deviennent difficiles ou impossibles. Avec les liquides de Flemming non acétifié, de Champy ou de Benoit, elle pourra durer quelques minutes. Le matériel est ensuite lavé sérieusement à l'eau distillée, puis inclus selon la technique de E. Chatton (1927), en opérant de la manière suivante.

Les Euglènes triées à la pipette, en suspension dans la plus petite quantité d'eau possible, sont placées sur un porte objet recouvert d'une couche d'un millimètre d'agar (gel à 1·3%, étalé à chaud, puis gardé jusqu'à l'emploi, à froid, en chambre humide). L'eau est alors retirée à la pipette, ou par évaporation. A l'aide d'une très fine aiguille de verre ou de platine, les Flagellés sont rassemblés et orientés, si besoin est, en un petit amas rectangulaire. On laisse alors tomber sur lui, verticalement, de quelques centimètres, une goutte de gélose fondue, et non brûlante: elle emprisonne les Protistes et les fixe à l'agar sous-jacent. Une fois qu'elle est solidifiée, on rajoute quelques nouvelles gouttes de gélose pour obtenir une surface plane. La prise une fois faite, à l'aide d'un scalpel, on découpe le bloc en un rectangle semblable au tas des Protistes mais plus grand. L'excès de gélose est alors enlevé, puis je verse sur le bloc une goutte de solution de vert lumière dans l'alcool à 95°. Au bout d'une minute, le bloc coloré, et rendu ainsi plus facile à manipuler, est porté dans l'alcool absolu, où il sera déshydraté. Pour obtenir une déshydratation rapide et totale, j'utilise des tubes longs de 6 ou 7 centimètres, remplis d'alcool absolu, et dans lesquels les pièces à déshydrater sont obligées à demeurer en surface. Pour un amas de Protistes ne dépassant pas dans son ensemble 4 × 1 × 0·5 mm., la déshydratation est ainsi achevée, dans le même alcool, en une demi-heure. La pièce est mise dans le toluène pur, sur lequel elle flotte. Elle doit descendre au fond du liquide, en devenant transparente, en moins de 5 minutes. Le tube qui la renferme est alors mis quelques instants à l'étuve, puis la pièce est transférée dans la paraffine fondue. Un seul bain est suffisant, d'une durée moyenne d'une heure, mais que l'on peut prolonger sans inconvénient.

La coupe se fait normalement, mais, pour obtenir des rubans, il faut, en taillant la paraffine, laisser un peu de paraffine en avant, comme en arrière du bloc de gélose. Les coupes peuvent, par cette technique, être exécutées plus minces qu'à la paraffine seule.

Cette technique ne présente pas de difficultés spéciales; elle offre une cause d'insuccès: si la déshydratation n'est pas suffisante, la gélose se ratatine au moment où la pièce, imprègnée de toluène, est mise dans la paraffine chaude. En pareil cas, du reste, le matériel n'est pas perdu: enlevant l'excès de gélose ratatinée qui l'entoure, on peut le réinclure dans un nouveau bloc d'agar, et recommencer.

Les colorations ont été faites soit à l'hématoxyline au fer, soit à l'Altmann, ce dernier étant régressé au différenciateur de Volkonsky. Avant cette dernière méthode, il est toujours avantageux de chromiser sur lame, c'est à dire de laisser les coupes, déparaffinées, de 12 à 24 heures dans la solution saturée de bichromate de Potassium, et de colorer après lavage rapide.

(*b*) *Les Euglènes incolores*

Cinq souches ont été étudiées, dont E. G. Pringsheim a donné, plus haut, l'histoire: trois (nos. 1, 2 et 4, p. 68) dérivent de formes apigmentées, découvertes comme telles. Les deux autres souches, nos. 5 et 6, ont été obtenues en dérivation des souches vertes nos. 4 et 7, ainsi qu'il a été expliqué plus haut (pp. 59, 60 *et sqq.*).

Ainsi qu l'a indiqué déjà E. G. Pringsheim, toutes ces souches sont morphologiquement parlant, très voisines: forme générale plus ou moins allongée, position médiane ou postérieure du noyau, taille du flagelle.

Partout, le système flagellaire est identique: deux racines flagellaires dont l'une est plus épaisse que l'autre, partant chacune d'un cinétosome unique ou dédoublé, convergeant plus ou moins vite, et paraissant se confondre dans le goulot. Ni de stigma, ni de photocepteur.

L'étude par les colorants vitaux ne révèle rien au niveau de la cuticule, mais seulement un vacuome banal, plus profond.

L'étude cytologique sur coupes montre dans toutes les souches les dictyosomes, tels qu'ils ont été décrits par A. Hollande, après que je les ai eu démontrés chez les Volvocales. Ils sont plus ou moins abondants, plus ou moins faciles à colorer.

En ce qui concerne les constituants décelés par les méthodes mitochondriales, voici les caractéristiques de chaque souche.

Souche no. 4. Variations individuelles importantes, qui se graduent selon une série, concernant un constituant unique, à réactions mitochondriales, dont les aspects sont les suivants: filaments grêles, ramifiés et anastomosés en un réseau, principalement périphérique (Fig. 4*d*), cordons ou rubans plus ou moins élargis, généralement ramifiés, tantôt continus (Fig. 4*a*, *b*), tantôt égrenés en courts éléments abondants et bien séparés (Fig. 4*e*).

Fréquemment, certaines ramifications de ces rubans plats se prolongent, en profondeur par des filaments minces (Fig. 4*c*). On note aussi une tendance fréquente des rubans ou filaments à s'orienter en spirale sénestre, correspondant à celle des stries cuticulaires.

Il n'existe aucune différence de réaction, ou de colorabilité, entre ces divers éléments, qui ne se distinguent que par leur aspect général, conduisant à les qualifier, soit de ***réseau mitochondrial***, ou de chondriocontes, quand ils sont grêles, soit de ***réseau leucoplastique***, ou de leucoplastes, quand ils sont aplatis en rubans. Tous ces éléments sont incontestablement d'origine identique; puisqu'ils ont les mêmes réactions de coloration, et que celles-ci correspondent à celles du chondriome, nous inclinons à faire nôtre l'opinion de A. Hollande, qui, seul jusqu'alors, les a assimilés au chondriome. L'étude des formes vertes nous permettra, plus bas, de nous prononcer avec certitude (p. 74).

Souche no. 1. Très analogue; mais l'aspect 'réseau leucoplastique' (Fig. 5*a*, *d*) est plus fréquent que l'autre, tout du moins en surface; en profondeur, par contre, c'est l'aspect 'réseau mitochondrial' qui domine; certains individus montrent un réseau mixte (Fig. 5*b*, *c*).

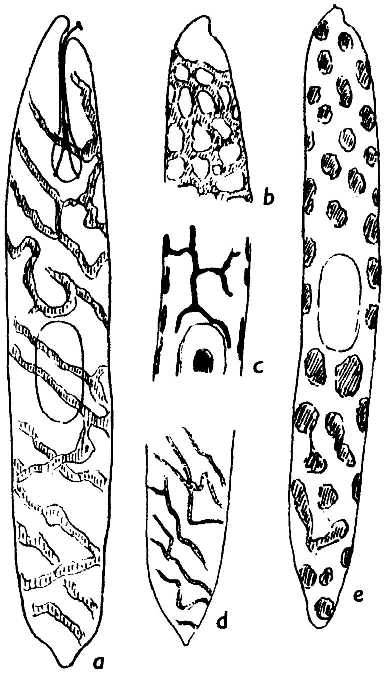

Fig. 4

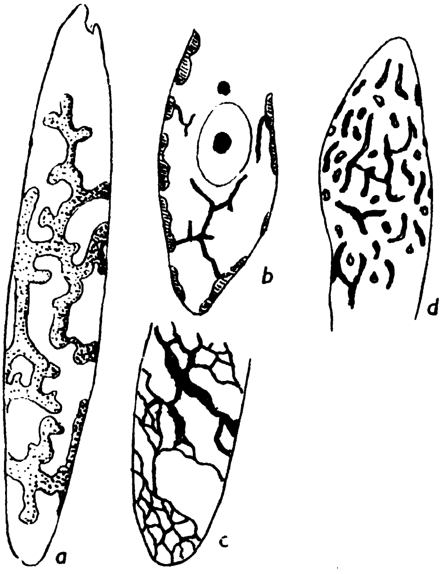

Fig. 5

Fig. 4. *Euglena gracilis* incolore, souche 4: *a*, *b*, *e*, aspects superficiels du chondriome, type 'leucoplaste'; *d* et *c*, vue superficielle et profonde, de l'aspect 'réseau mitochondrial'; en *c* continuité entre le 'leucoplaste' superficiel et le réseau profond. Appr. × 1500. Flemming fer.

Fig. 5. *Euglena gracilis* incolore, souche 1 : *a*, *c*, *d*, vues superficielles du type 'leucoplaste'; *c*, type mixte; *b*, vue profonde, réseau mitochondrial, en continuité avec le soit-disant leucoplaste. × 1500. Flemming fer.

Souche no. 5. Très voisine (Fig. 6), bien que ce soit l'aspect 'réseau mitochondrial' qui se rencontre habituellement (Fig. 6*a*), se suivant alors dans toute la masse cellulaire. L'autre aspect, avec réseau très lâche, est toujours très superficiel (Fig. 6*b*). Le réseau mitochondrial paraît d'autant plus développé qu'il y a davantage de paramylon.

Souche no. 6 (Fig. 7). L'aspect réticulé est ici moins fréquent: la cellule est garnie d'éléments ayant l'aspect de chondriocontes isolés. En surface cependant, on note une tendance à l'élargissement, à l'étalement, vers l'aspect leucoplastique mais, par segments indépendants (Fig. 4*a*, *b*). En profondeur, souvent les batonnets s'égrènent en mitochondries—au sens strict du mot—(Fig. 4*c*). Il n'y a donc jamais de rubans aussi nets que dans les types précédents. Fréquemment aussi, on rencontre quelques gros globules profonds, sidérophiles.

Souche no. 2 (Fig. 8). Assez différente des précédentes: le réseau colorable est ici plus complexe, aussi bien superficiel que profond, avec cordons irréguliers non aplatis en rubans, mais de section arrondie, s'élargissant en nœuds, mal distincts du peu de cytoplasme indifférencié, intercalé entre les nombreux grains de paramylon. A première vue, l'aspect du cytoplasme évoque celui d'une émulsion de grains de paramylon dans une masse indifférenciée. Il faut un examen attentif pour reconnaître dans cette masse l'équivalent du réseau des autres formes (Fig. 8*a*).

En surface, et aux nœuds de ce réseau, de petites sphères régulières se colorent intensément. Tantôt homogènes, il arrive aussi qu'elles paraissent creuses, se montrant centrées par une ou deux vacuoles claires (Fig. 8*b*, *c*).

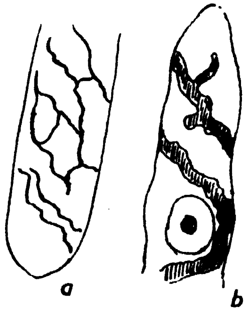

Fig. 6

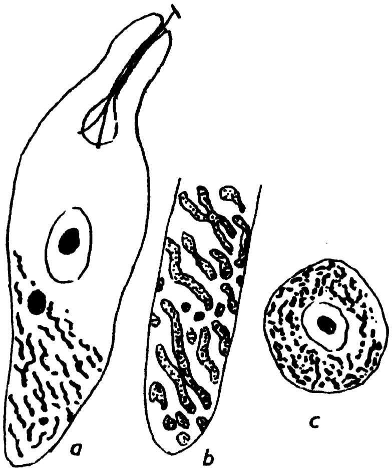

Fig. 7

Fig. 6. *Euglena gracilis* incolore, souche 5; vues superficielles. *a*, type réseau mitochondrial; *b*, type 'leucoplaste'. × 1500. Flemming fer.

Fig. 7. *Euglena gracilis* incolore, souche 6: *a* et *b*, vues superficielles; *a*, aspect mitochondrial; *b*, aspect 'leucoplaste'; *c*, coupe transversale: chondriocontes et mitochondries (*sensu stricto*). × 1500. Flemming fer.

Ces sphérules sont strictement corticales, et souvent disposées en lignes inclinées sur l'axe, à peu près régulières. Cette disposition suggère leur assimilation aux corps mucifères ou aux trichocystes, de forme et de disposition si caractéristiques chez de nombreuses espèces d'Euglènes vertes ou incolores. (Cf. A. Hollande, 1942, p. 151 *et seq.*)

Toutefois, ces sphérules ne se colorent pas par les réactifs vitaux, pas plus que par l'Iode; serrées sous le microscope, elles n'émettent ni mucus, ni filament; leur nature reste ainsi à préciser.

Elles m'incitent à considérer cette souche comme spécifiquement distincte des précédentes.

En somme, les Euglènes incolores examinées possèdent toutes, indépendemment de leur Golgi et de leur vacuome, un troisième constituant cytoplasmique, dont l'aspect peut, selon les tendances, être ramené à celui d'un ensemble de mitochondries, ou d'un ensemble de leucoplastes, mais dont les propriétés tinctoriales sont celles du chondriome.

(*c*) *Les Euglènes vertes*

Quatre souches ont été étudiées, dénommées: 7, 9, 4, 6 (cf. p. 58).

Toutes présentent les caractères normaux de l'espèce *Euglena gracilis* Klebs, et diffèrent des formes incolores examinées précédemment, par l'existence des chloroplastes, d'un stigma, avec, en vis à vis, sur la plus grosse des deux racines flagellaires, un photocepteur typique.

Les deux premières possèdent régulièrement des 'corps bruns', tels que les a définis C. L. Baker: sphérules brunes, plus ou moins régulièrement agglomérées, bleuissant par l'Iode, variables selon les individus et les conditions de culture.

L'étude mitochondriale est malaisée quand les cellules sont bourrées de paramylon: à ce point de vue, la souche 7, qui paraît en fabriquer moins que les autres, est la plus propice à l'examen. Les plastes, imprégnés ou non de chlorophylle, ressortent plus nettement après une fixation d'une demi-heure au liquide de Bouin normalement acétifié, qui atténue ou supprime la colorabilité du chondriome.

Souche 7 (Fig. 9*a*, *b*). En plus des chloroplastes typiques, représentant donc le plastidome, centrés par leurs pyrénoïdes, il existe un chondriome indiscutable, si facile à mettre en évidence, que je ne puis m'expliquer l'échec total qu'ont enregistré à son sujet mes prédécesseurs, y compris A. Hollande. C'est un réseau typique formé de filaments grêles et calibrés, dessinant des mailles irrégulières dans toute la cellule, et tout autour des chloroplastes. Ce réseau présente de nombreuses extrémités libres, qui peuvent se résoudre en chondriocontes, ou même en mitochondries (*s.s.*). Sa colorabilité n'est pas rigoureusement homogène, mais le montre constitué de segments bactéroïdes, unis par une matière moins colorable ou achromatique.

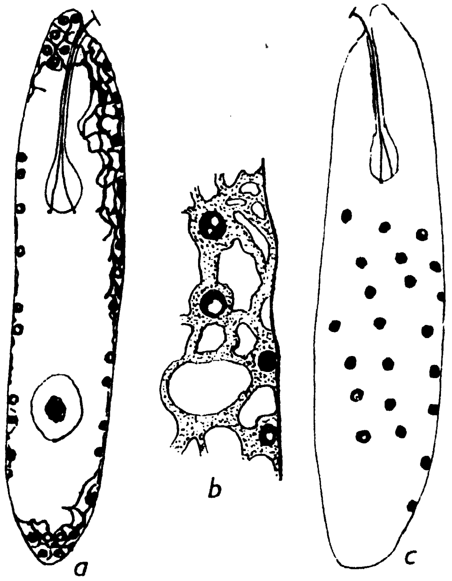

Fig. 8. *Euglena gracilis* incolore, souche 2: *a*, coupe axiale, réseau mitochondrial et sphérules; *b*, sphérules et réseau en surface, × 3000 *c*, disposition des sphérules en surface, × 1500; Flemming-fer.

Il correspond ainsi au réseau mitochondrial typique des Péridiniens, que j'ai signalé en premier lieu en 1923, qui a été ensuite décrit en détails par B. Biecheler (1934), que M. Chadefaud (1935) a étudié chez de nombreuses Algues et que j'ai reconnu également chez les Volvocales (1937–8), où A. Hollande l'a ensuite retrouvé.

Au niveau des chloroplastes, il arrive que des filaments de ce chondriome viennent se disposer en un cercle assez régulier autour des pyrénoïdes (Fig. 9*c*), tandis que d'autres bordent la périphérie des plastes. Il parait vraisemblable que de tels rapports de position ne soient pas uniquement fortuits, et qu'ils soient l'indice d'une coopération fonctionnelle. L'apparition des grains de paramylon, signalée toujours au contact des pyrénoïdes, qu'ils recouvrent en calottes, se produit donc tout aussi bien au voisinage immédiat du chondriome.

Souche 9 (Fig. 10). Très voisine, malgré que le chondriome soit plus difficile à mettre en évidence. Même disposition des filaments du réseau autour des pyrénoïdes. A noter que les dictyosomes sont ici particulièrement faciles à colorer; ils paraissent aussi plus nombreux que dans toutes les autres souches étudiées, vertes ou incolores. Certains, doubles, ont la forme en X, les deux croissants étant disposés convexité contre convexité.

Souche 6. Très analogue à tous points de vue, mais l'abondance du paramylon rend gènante l'étude cytologique.

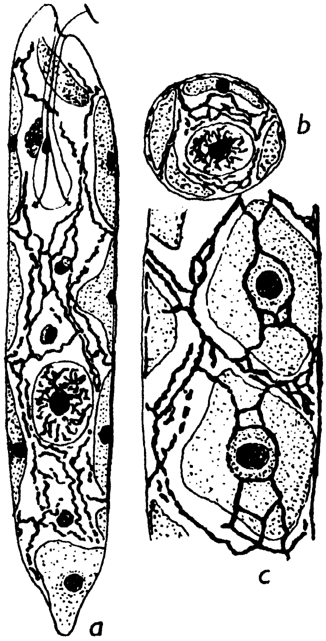

Fig. 9

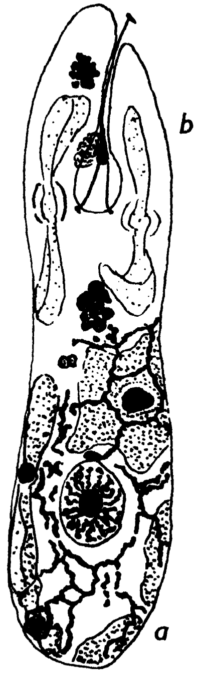

Fig. 10

Fig. 9. *Euglena gracilis* verte, souche 7: *a*, section axiale; chromatophores et pyrénoïdes; réseau mitochondrial profond; stigma, photocépteur, trois dictyosomes; *b*, section transversale au niveau du noyau, chromatophores et réseau profond, × 1500; *c*, vue en surface du réseau mitochondrial et des chromatophores, × 2000. Flemming fer.

Fig. 10. *Euglena gracilis* verte, souche 9: *a*, moitié postérieure, chondriome et chromatophores, deux dictyosomes; *b*, moitié antérieure, aspect *in vivo*: deux amas de 'corps bruns', chromatophores avec calottes de paramylon. Stigma et photocépteur. × 1800.

Souche 4. Voisine également, mais la forme des chloroplastes est moins régulière: ils sont souvent déchiquetés à leur périphérie, ou allongés en rubans simples, ou bifurqués en Y. Leurs pyrénoïdes sont plus petits, l'aire claire qui les entoure étant plus vaste.

Indépendemment du Golgi et du vacuome, ces quatre souches vertes renferment donc *deux* constituants cytoplasmiques bien distincts et caractéristiques, c'est à dire *un de plus* que les formes incolores.

Comme ce constituant supplémentaire est un plastidome chlorophyllien, homologue de celui des plantes supérieures, l'autre ne peut être que le chondriome. Il faut donc bien considérer comme telle la nature de celui que nous hésitions à dénommer, pour des raisons de forme, chez les types incolores (cf. p. 71).

(*d*) *Chondriome et plastidome; la disparition momentanée de la chlorophylle*

Chez tous les types étudiés de *Euglena gracilis*, qu'ils soit verts ou incolores, existe donc un chondriome: c'est l'élément reconnu comme tel par A. Hollande chez les formes incolores. Ni Brown, ni Baker ne l'avaient reconnu dans la forme verte, où il a échappé également à A. Hollande. Très vraisemblablement aussi, M. Chadefaud ne l'a pas vu non plus chez *E. anaboena*. Comme les Péridiniens pigmentés et les Volvocales vertes le chondriome se présente ici sous forme d'un appareil réticulé, indépendant des chloroplastes.*

Chez les formes dont la chlorophylle a disparu définitivement, il n'existe plus de plastidome: la cellule ne renferme alors plus qu'un chondriome gardant son allure normale, ou évoluant vers ces aspects auxquels les premières formes étudiées nous ont habitués, et qui par leur étalement superficiel, rappellent un appareil plastidial.

Le prétendu 'leucoplaste' des formes incolores n'est donc nullement l'homologue de celui des plastes décolorés des Phanérogames: ce n'est qu'un *aspect* du chondriome.

E. gracilis verte, grâce à ses changements réversibles de pigmentation, selon qu'elle est maintenue à la lumière ou à l'obscurité, nous offre en outre la possibilité de comparer l'évolution de ses plastes véritables dans l'une ou l'autre de ces conditions.

C. L. Baker a abordé l'étude cytologique de cet intéressant problème, examinant le plastidome d'Euglènes ayant effectué des séjours de durée variable à l'obscurité.

Dans un milieu tel que la multiplication des Flagellés ne soit pas arrêtée par le manque de lumière, il constate une réduction du nombre des plastes, qui peuvent se fragmenter, devenant le 'leucoplaste', ensemble d'éléments irréguliers petits, très colorables, dont le nombre diminue avec le temps. Au bout de 7 mois, ce leucoplaste a presque totalement disparu.

Baker n'ayant pu nous renseigner sur les changements simultanés du chondriome, j'ai refait son expérience sur les souches 7 et 9.

Deux séries de cultures ont été suivies toutes deux jusqu'à 3 mois de séjour à l'obscurité: mes résultats confirment en gros ceux du savant Américain.

La première série, contrôlée uniquement par les techniques mitochondriales, m'a renseigné principalement sur le chondriome, dont les modifications sont aussi surprenantes qu'inattendues: la partie superficielle du réseau, exclusivement constituée à la lumière par des filaments grèles (Fig. 9*a*, *c*), change entièrement d'aspect, s'hypertrophie, s'aplatit en rubans qui s'appliquent contre la membrane, exactement comme ceux des formes incolores (Fig. 11 *b*, *c*). Ces cordons peuvent également s'enrouler en spires sénestres. Ils se continuent en profondeur par un réseau grêle et de faible densité.

Les plastes, maintenant incolores, ont subi des modifications déjà en partie reconnues par Baker, bien que moins rapides dans la souche 7 que dans celles qu'il a lui-même étudiées.

Les leucoplastes passent en profondeur, s'approchant souvent du noyau, pouvant s'agglutiner plus ou moins à son abord. Après fixation mitochondriale, ils paraissent souvent demeurés aplatis, parfois tendus entre les mailles du réseau mitochondrial (Fig. 11*a*). Après fixation au liquide de Bouin, ils sont le plus généralement globuleux, ovoïdes, ou étirés en fuseaux (Fig. 12*b*, *c*). Très peu montrent encore des pyrénoïdes reconnaissables (1 pour 300 ou 400). Chez les autres, on ne voit que des grains sidérophiles ou des batonnets, toujours plus petits que les anciens pyrénoïdes. Leur position est généralement excentrique, souvent tout à fait terminale.

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* Il existe un chondriome de même type chez *Euglena proxima*: il est vraisemblable qu'il en est de même chez beaucoup d'autres Euglènes.

Il semble que la substance même des pyrénoïdes ait ainsi subi une véritable dispersion.

Ces dernières notions ont été acquises par la seconde série d'expérience qui m'a permis en outre de suivre le nombre des plastes, constatant qu'il est loin de varier uniformément chez tous les individus, pas plus après un mois qu'après trois mois d'obscurité. Le plus souvent, il subsiste une dizaine de plastes: leur nombre est donc resté sensiblement le même qu'à la lumière.

Mais il est une minorité d'Euglènes, environ 2%, qui sont loin d'atteindre ce nombre, qui paraissent même ne plus avoir aucun plaste, autant, tout du moins qu'il est possible d'en juger d'après l'examen des coupes. Comme chaque individu fournit deux ou trois coupes, et qu'il est pratiquement impossible de les retrouver avec certitude sur les préparations, il n'est pas possible d'affirmer ainsi la disparition totale des plastes.

Seule la méthode des cultures permettra de régler cette question.

J'ajouterai que je n'ai jamais constaté de fragmentation des plastes, et je pense que Baker a dû être parfois induit en erreur par le chondriome, dont il subsiste presque toujours des restes après les fixations ordinaires (Fig. 12*b*, *c*), une grande partie de ce que l'on peut considérer comme le 'réseau de fond' du cytoplasme n'étant pas autre chose que le chondriome altéré par la fixation et restant partiellement colorable.

L'obscurité ne modifie pas du tout la cinétide. Pour ce qui est du stigma, j'ai noté sur le vif que certains individus n'ayant pas reverdi, en possèdent un, très réduit, d'autres, au contraire, ayant plusieurs taches de carotène éparses dans la cellule.

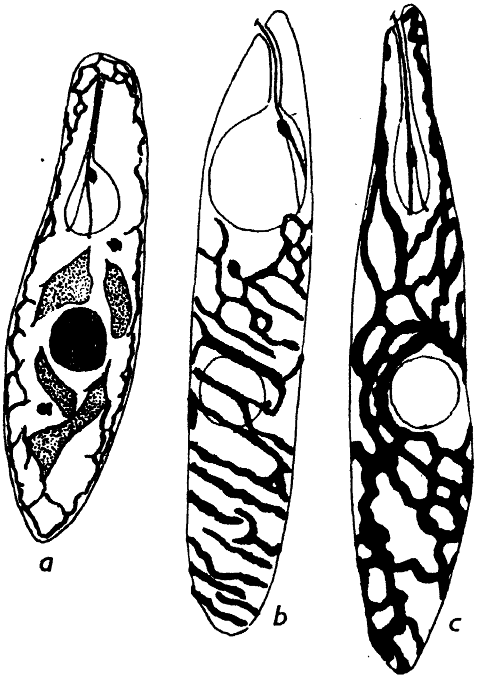

Fig. 11. *Euglena gracilis* verte, souche 7 décolorée par séjour de 3 mois à l'obscurité: *a*, coupe axiale avec leucoplastes vrais; *b* et *c*, vues superficielles du chondriome hypertrophié (comparer à la Fig. 9, p. 74). × 1500. Flemming fer.

Au retour à la lumière, le verdissement réapparaît dans la masse, mais d'une manière inégale selon les individus. Les premières cellules qui montrent de la chlorophylle me paraissent être principalement des individus sphériques, peu mobiles, ou immobiles. Il est difficile de dire s'il ne s'agit pas d'individus qui ont passé la période d'obscurité sous kyste, accolés aux parois des récipients, et dont les plastes n'ont subi aucune involution. Au moment de remettre mes cultures décolorées à la lumière, j'ai, dans l'une d'elle, séparé par un changement de tube à essai, la portion périphérique, c'est à dire fixée à la paroi, ou rampant sur elle, de la portion centrale, correspondant aux individus les plus actifs. Le premier de ces deux tubes a été aussi le premier à reverdir: au bout de 24 heures l'ensemble des individus est vert. Dans le second, au contraire, nombreux sont les Flagellés qui n'ont pas récupéré leur pigment au bout de 6 jours.

Dans les conditions habituelles, de tels individus apparaissent mal, noyés qu'ils sont dans la masse des individus verts, qui, avantagés par la lumière, se multiplient intensément, si la température le permet. Le froid ralentit, en effet, considérablement la vitesse de reverdissement, ce qui ne peut guère s'expliquer que par une action sur la vitesse de multiplication.

En conclusion, il y a donc chez les Euglènes vertes provisoirement décolorées à l'obscurité, changement d'aspect de leur chondriome qui devient identique à celui des Euglènes incolores. On conçoit ainsi qu'il suffit à ces Euglènes vertes de perdre leur plastidome pour s'identifier aux Euglènes incolores, chez lesquelles, faute de plastidome, tout reverdissement est devenu impossible.

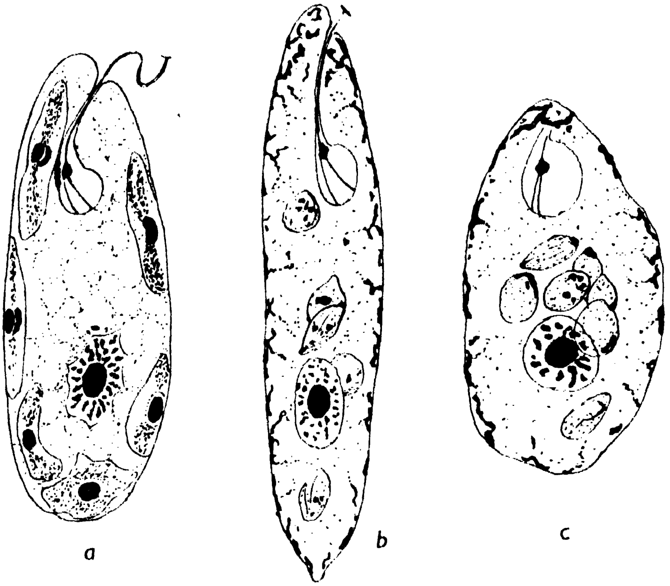

Fig. 12. *Euglena gracilis* verte, souche 7; *a*, exemplaire maintenu à la lumière; *b* et *c*, 3 mois à l'obscurité. Involution des plastes; résidus de chondriome. × 1500. Bouin fer.

On sait que chez les Phanérogames, la théorie de W. Schimper et A. Meyer, d'abord quelque peu battue en brèche par les premières recherches de Guilliermond, a été ensuite confirmée par cet auteur aidé de ses deux élèves, Emberger et Mangenot. Chez les Ptéridophytes et chez les Algues, ces deux derniers savants ont démontré que plastes et chondriosomes constituent deux catégories d'organites dont l'évolution est rigoureusement séparée, et qui, chacune, possèdent la continuité génétique.

Les Euglènes ne constituent pas un cas particulier: la même règle s'applique pour elles.

Cependant, la malléabilité du chondriome se révèle, chez *Euglena gracilis*, considérable et telle que, si le plastidome perd momentanément sa fonction assimilatrice ou disparait, pour quelque raison que ce soit, le réseau mitochondrial s'en montre affecté, et prend des aspects qui s'observent d'une manière normale et permanente chez les Euglènes qui ont perdu toute trace de plastidome.

L'interprétation de ces aspects étalés comme 'leucoplaste', ne peut être soutenue: il s'agit d'une simple convergence morphologique entre organites cellulaires distincts, ainsi que le démontre formellement l'étude des *E. gracilis* décolorées à l'obscurité, et qui, en plus de ce chondriome étalé, possèdent encore un leucoplaste véritable.

L'étude cytologique comparative des *E. gracilis* vertes et incolores montre donc que les différences morphologiques reconnaissables entre ces deux catégories de formes, sont liées essentiellement aux différences de leurs modes de nutrition. En effet, si les types verts sont placés à l'obscurité, dans les mêmes conditions de nutrition que les types incolores, leur organisation cellulaire devient presque la même que celle de ces derniers.

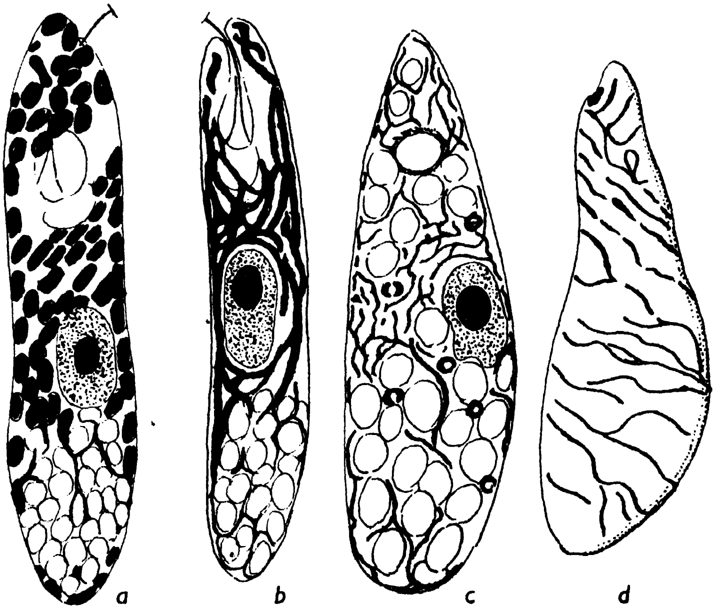

Fig. 13. *Euglena gracilis* incolore; *a* et *b*, culture sur terre-blé; coupes. *c* et *d*, peptone-acétate de Sodium: *c*, coupe axiale; *d*, vue en surface. En *c*, nombreux dictyosomes. Flemming-Altmann.

(*e*) *Action du milieu de culture sur la disposition et l'importance du chondriome*

Les aspects variés du chondriome, dans une même souche, tels que nous les avons reconnus précédemment (cf. pp. 70 *et sqq.*), doivent-ils être considérés comme fixés, c'est à dire, comme dépendants d'une cause génétique, ou bien, comme un simple phénomène somatique, adaptation directe de la cellule à son milieu?

M. Volkonsky a résolu ce problème dès 1930 chez *Polytoma uvella*, montrant que la seconde hypothèse y est la bonne. La formation qu'il désigne comme 'leucoplaste', et qui est en réalité le chondriome, varie en fonction des sources d'Azote de la cellule. Il est développé au maximum dans les milieux à l'acétate d'Ammonium, au minimum dans ceux à base de peptones.

J'ai repris cette étude sur les Euglènes incolores.

Une même souche d'*Euglena gracilis* incolore (*Astasia longa*) a servi à ensemencer des tubes de milieu terre-blé (Pringsheim, 1946*b*), ainsi relativement pauvres en Azote assimilable, d'une part, et peptone-acétate de Sodium, riches en Azote, d'autre part. Dans les deux milieux la multiplication a été poursuivie pendant 12 jours à température constante de 18°, puis tous ont été traités par la même technique.

Le résultat est frappant (Fig. 13*a–d*).

Dans le premier milieu, les individus restent élancés (*a* et *b*); leur chondriome continu ou non, est épais, sauf au milieu des grains de paramylon, localisés au pôle postérieur, et peu abondants. On note d'importantes variations dans la forme même du chondriome, les figures *a* et *b* étant deux extrèmes d'une série.

Dans le second milieu, l'ensemble est extrèmement homogène: les individus sont épais, à extrémité arrondie, très riches en paramylon. Leur chondriome est partout très grèle, et en réseau discontinu, aussi bien en surface (*d*), qu'en profondeur (*c*). Les dictyosomes sont très abondants.

On peut donc élargir aux Euglènes incolores les conclusions de Volkonsky.

En outre, ces faits, joints à ceux des pages précédentes, accentuent le caractère de plasticité que présente le chondriome dans toutes ces formes. Il semble que l'on se fasse une conception beaucoup trop statique de celui-ci: loin de constituer un élément fixe dans la cellule, le chondriome y est extrèmement variable, son aspect et son volume dépendant de l'état physiologique et des conditions de milieu.

VI. DISCUSSION

(*a*) *Cytological aspects*

The disappearance of the chromatophores and of the eye-spot, and the relation between these two phenomena, raise certain questions which have not yet been envisaged. Lwoff, for instance, repeatedly puts forward the view that starch-like reserves are only found in the presence of plastids (1932, p. 130; 1943, p. 110), and that colourless organisms producing such reserves always possess leucoplasts. He considers that these leucoplasts have been demonstrated in *Polytoma* by Volkonsky (1930) and Provasoli (Lwoff & Provasoli, 1935) and in *Polytomella* by Rabinovich (1940). I expressed doubts (1937, pp. 71 *et seq.*) as to the correctness of Lwoff's statements, but his new book (1943) repeats the old theory without furnishing new facts and, without discussing my detailed criticism, rejects it in a few words.

The apochlorotic form of *Euglena gracilis* is especially suitable for an experimental approach to the problem. The chromatophores disappear, the formation of paramylon continues. Although Lwoff (1932, p. 132) mentions *Astasia* among the 'formes dépourvues de plastes', he nevertheless classes it, together with *Polytoma*, *Chilomonas*, etc. among his leucophytes which are characterized by possessing leucoplasts. He would therefore certainly expect that the colourless form of *Euglena gracilis* would possess leucoplasts, since it deposits paramylon in the same way as the green form. He actually claims (1932, p. 130) 'Parmi les espèces sans chlorophylle possédant un plaste on peut citer...les Euglènes incolores', not envisaging the possibility that his *Astasia chattoni* (syn. *A. longa*) might be a colourless *Euglena*. Lwoff's attitude is underlined by the remarks on *E. Mesnilii* (Lwoff & Dusi, 1935; Lwoff, 1943, pp. 78, 84 *et seq.*). In cultures of this species grown in the dark, cells with a reduced number of chromatophores were found, this reduction sometimes leading to the presence of cells without chromatophores. Lwoff emphasizes that the loss of the plastidome is accompanied by loss of the capacity to form paramylon. In spite of the fact that this disharmony in cell and plastid division is altogether comparable to Ternetz's explanation for the loss of chromatophores in *E. gracilis*, which is apparently accepted by Lwoff, he supposes that the latter retains leucoplasts for the deposition of paramylon. Lwoff & Dusi did not succeed in rearing ooplastidic subcultures of *E. Mesnilii* which alone would have shown whether the lack of paramylon was due to the loss of the plastidome.

Prof. Hovasse obligingly agreed to investigate my strains of *E. gracilis* with the elaborate cytological methods of the French school, which he has further improved. In all the apochlorotic strains he found a network staining like a chondriome, but nothing of the nature of plastids, while in the green strains similar, but fainter and less substantial structures were observed. Prof. Hovasse therefore abandons the view that this network can be of the nature of a leucoplast. Such an interpretation is also unsuitable because the true leucoplasts of *E. gracilis*, as are formed in the dark, have quite a different appearance, being separate, disk-shaped yellowish structures, similar to the chloroplasts though smaller (Zumstein, 1900, p. 160; Baker, 1933, p. 444).

There are several indications that it is not the plastidome itself that is primarily affected by some kind of destructive agent. The consecutive disappearance of the chromatophores, as well as of the eye-spot, seems to be due to an inner disposition of the cell, which affects both these organs in the same way, rather than to an immediate influence from outside. They disappear without leaving any trace, although the cell remains vigorous and capable of multiplication. The chondriome, having more space at its disposal, can develop in a more extensive manner than in green individuals (cf. p. 77). The connexion between chondriome and paramylon is entirely hypothetical. In *Polytoma* Volkonsky (1930) observed alterations of the chondriome in connexion with changes in nutrition, and these were believed to indicate a relation between the 'leucoplasts' and the deposition of reserves. In *Polytomella* no such phenomena were observed (Rabinovich, 1940, p. 435), but in *Astasia longa* Prof. Hovasse has found them without indication of a relation to paramylon deposition.

Prof. Hovasse has proved that the plastidome and the eye-spot of *Euglena gracilis* have finally been lost during the process of transformation into the colourless form. This could be taken as an indication that the eye-spot is part of the plastidome or, as Rothert (1914) claimed, is really a chromoplast. The question therefore arises whether the latter is deposited in a special plasmatic structure which might persist after the disappearance of the colouring matter. To my mind no proof of the existence of such a structure has so far been given, and it is not to be found in the hyaline form. Moreover, the interpretation of the eye-spot as a chromoplast is opposed by other facts. Quite a number of apochlorotic flagellates have retained eye-spots (Pringsheim, 1937) which in them have not shared the fate of the chromatophores. There is also no instance known, in which a cell possesses more than one kind of plastid, their modification seemingly depending on the general conditions in the cell; and not only the eye-spot but also the flagellar thickening disappears with the chromatophores, although this cannot possibly be a part of the plastidome. The pigmented portion and the flagellar thickening are better taken both as parts of the light-sensitive organ of the cell (Mast, 1938), the connexion being functional rather than morphological.

(*b*) *Ecological aspects*

Ternetz's (1912, pp. 437, 490) view that the green form of *E. gracilis* would alone be found in nature seemed well founded, since the hyaline race apparently has no advantage over the green one in respect of competition and should multiply more slowly under natural conditions. The few individuals arising under certain conditions, as observed by Ternetz, are soon suppressed and no trace is left. Only artificially isolated colourless cells can give rise to populations of this race. Ternetz drew the logical conclusion that this does not happen in nature, and this conclusion was the more warranted since she found that the hyaline race was difficult to maintain (p. 487).

This conclusion has nevertheless proved to be mistaken. Colourless populations from natural habitats have repeatedly been recorded under the name of *Astasia longa*, persisting long enough to develop into large populations. Ternetz's reasoning must therefore be faulty. The apochlorotic race in fact multiplies well when suitably nourished and is inferior to the green race only when the latter is living phototrophically. The most important fact is, however, that the loss of chromatophores is probably caused by mutation with an additional influence of certain external factors, probably a deficiency of some kind, the nature of which is not known.

The fact that in nature green populations do not contain colourless cells, and colourless populations do not contain green cells, is certainly remarkable. It can be related to the rare occurrence of apochlorotic cells in green cultures. In this connexion the question arises whether the colourless form has originated rarely or even only on a single occasion, then spreading to other localities, or whether it frequently arises from the green form, perhaps wherever it occurs in nature. Benecke (1900, p. 559), discussing the origin of apochlorotic from pigmented organisms in connexion with his observations on colourless diatoms, expresses the opinion that 'their derivation took place long ago, and that the establishment of the colourless forms was favoured by suitable habitats'. Although the latter part of this sentence is in line with observations on *Euglena gracilis*, the first part does not agree with what has been found in this species. The appearance of apochlorosis is an event restricted to limited periods, but has probably occurred often. Although new habitats may be colonized by spreading, most of the populations of *Astasia longa* are probably derived from the multiplication of cells left over from a previous colourless population.

(*c*) *Taxonomic aspects*

The instance furnished by *Euglena gracilis* and its apochlorotic race is of great taxonomic interest, first because of its bearing on the interrelationship between green and colourless Euglenineae, and secondly because it is perhaps the first demonstration that two species (*E. gracilis* and *Astasia longa*) referred for generally accepted reasons to different families, can actually change one into the other, not only under laboratory conditions but also in nature. If Calkins's (1933) view were accepted their respective positions in the taxonomic scale would even be farther apart, since he refers *Euglena* to the vegetable and *Astasia* to the animal kingdom.

As pointed out by Zumstein (1900, p. 151), the occurrence of colourless forms in *Euglena* removes the main difference between this genus and *Astasia*. Actually no other general and practicable means of differentiation are known. Even Klebs, on whom the later writers rely for the distinction that *Euglena* divides after shedding the flagellum, while *Astasia* is supposed to keep it, does not see in this a trustworthy taxonomic feature. He says 'Im wesentlichen ist die Organisation der Astasien dieselbe wie die der hyalinen Euglenen; die Trennung der beiden Gruppen kann nur eine vorläufige sein, einmal weil der Unterschied in der Theilungsart nicht sehr gross ist, und ferner, weil es auch noch nicht bewiesen ist, dass er ganz allgemein ist, da von einigen Euglenen die Theilung noch nicht bekannt ist.' (Klebs, 1883, p. 322.)

Some authors (Zumstein, 1900, p. 155; Moroff, 1904, p. 103; Smith, 1933, p. 612) have taken the presence of an eye-spot as an indication that a form belonged to *Euglena* and not to *Astasia*; but in such instances as *Monas*, *Anthophysa*, *Polytomella*, *Polytoma*, *Tetrablepharis*, and *Hyalogonium* no one would accept the eye-spot as the sole generic distinction. Jahn & Kibben (1937) have referred the colourless Euglenineae with an eye-spot to a distinct genus *Khawkinea*, with *Khawkinea* (*Astasia*) *ocellata* (Khawk.) Jahn & Kibben as the type species. Such a segregation loses much of its force by virtue of the fact that *A. longa*, though certainly closely related to *Euglena*, lacks an eye-spot. The other features, foremost the flagellar thickening, on which Jahn & Kibben base their generic diagnosis, are so closely connected with the eye-spot that they disappear with it. Apart from the presence or absence of chromatophores, there remains thus, according to the American authors, only the bifurcation of the flagellum in *Euglena* and *Khawkinea*, and the simple flagellum of *Astasia* (and all Astasiaceae) as a means of distinction between the two series of forms. Since, however, Lackey (1934, p. 149) and Hollande (1942, p. 89) have demonstrated two flagellar 'roots' in certain members of *Astasia*, the value of this character is called into question. Whatever may be the position of other genera of Astasiaceae, *Euglena* and *Astasia* really appear to merge into one another (Ternetz, 1912, p. 436; Mainx, 1927, pp. 349, 351; Doflein-Reichenow, 1929; Pringsheim, 1936, p. 83) and can be separated only for practical reasons, as Klebs has already foreseen.

The other conclusion to be drawn from the study of *Euglena gracilis* is still more fundamental. It would be interesting enough if a colourless 'artificial' race of a green and facultatively autotrophic organism were found to thrive permanently under established heterotrophic conditions. The abundant growth of such a race in a natural habitat implies that both it and the green race are, so-to-say, 'good species', not given different names only for lack of better knowledge but both actually with a claim to taxonomic rank.

It is tempting to discuss further implications of this fact, such as the derivation of apochlorotic forms in other groups, but before more evidence has been collected such conclusions would not amount to much. It can, however, be said that the origin of a species has actually been traced.

Certain aspects remain uncertain. Thus only two out of more than a dozen strains from various places have produced colourless cells and even in these the phenomenon has been observed only during a short period at the beginning of the experiments. Had a well-defined factor been found to induce the origin of hyaline cells, the behaviour of the other strains could have been tested, but as it is, the problem as to whether there is in this respect an internal difference between the strains could not be solved.

It is therefore premature to expect an answer to the question as to the taxonomic status of *Euglena gracilis*, forma *hyalina* (syn. *Astasia longa*). Even serological investigations to not clarify the situation. Elmore-Sauer (1935) found that the hyaline could be differentiated from the green form by this means, but Lichtenstein (1922) had previously established that the dark form also is serologically different from the green light form.*

If it could be supposed that the cells, previously weakened by some influence, underwent degeneration of the chromatophores when affected by another external factor the change would be regarded as a modification. If the loss of plastids were due to a certain influence affecting cells previously altered by mutation, the position would already be different but it would still be best to speak of modification. If, on the other hand, only a gene-mutation is involved, the term 'variety' would alone be applicable. The last possibility is not likely to apply. It would not account for the occurrence of completely colourless populations in nature, if we reject the improbable suggestion that they originated from single cells which by chance multiplied after becoming separated from the bulk of their green relations and competitors as happens during the experimental isolation of apochlorotic cells.

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* Tanzer (1941) found by serological investigation that *Khawkinea halli* and *K. ocellata* are more closely related to *Euglena* (*gracilis*!) than to *Astasia* (*longa*!).

The instance afforded by the hyaline form of *Euglena gracilis* serves to clarify some conceptions regarding the phylogenetic origin of apochlorotic flagellates. Hall (1939*b*), in his review of 'The Trophic Nature of the Plant-like Flagellates', says: 'It is not impossible that, in an order containing colorless saprotrophic and holozoic types as well as chlorophyll-bearing species, some of the green forms may have evolved, or degenerated, toward the saprozoic method of nutrition' (p. 3). In my opinion certain Euglenineae owe their survival after loss of chromatophores to their ability to utilize organic compounds.

At another point (p. 10) Hall discusses: 'Whether certain species are photo-autotrophic or have really lost this primitive characteristic....' This sentence seems to me correct or incorrect according as the author uses autotrophic in the facultative or obligate sense. Strict autotrophy is probably not a primitive but a derived character compared with mixotrophy. It involves the inability to utilize organic substances present in the medium contrary to those formed within the cell.

The statement on the same page that '...evolution of colorless flagellates has been accompanied by fundamental changes in physiological characteristics' also receives some clarification through the study of *Euglena gracilis*. Changes in the physiology of nutrition need not 'accompany' the loss of assimilatory pigments, as a comparison of *Astasia longa* with the parent species shows. When a colourless species exhibits such changes, these have evidently taken place subsequently, either by further losses in the synthetic faculties, as for instance in other species of *Astasia*, or by morphological adaptations as in the Peranemaceae. The fundamental fact that a green species must needs be able to live without relying on photosynthesis if it is to survive the loss of its chromatophores, is clearly demonstrated by *Euglena gracilis*, but it could have been predicted on general grounds for all algae losing their pigments during the lifetime of individual cells. Only if the chromatophores would gradually diminish in size or depth of pigmentation, could the acquisition of the faculty to utilize organic substances have 'accompanied' the reduction in assimilatory equipment. There are not sufficient data to illustrate such an evolution (cf. Pascher, 1927).

The various instances of the loss of chromatophores have repeatedly been discussed (Pascher, 1916, 1917, p. 43; Lwoff, 1932, pp. 129–30; Meyer, 1933, p. 541; Fritsch, 1935, pp. 538, 541). Since there are no facts to add, I would refer only to the case of *Chlorella variegata*, investigated experimentally by Beijerinck (1904) and Meyer (1933) and compared both by the latter (pp. 200–1) and by Ternetz (p. 505) with *Euglena gracilis*. *Chlorella variegata* has shown the production of colourless cells for at least 29 years, but apart from this and from the fact that completely colourless substrains have not been obtained *C. variegata* behaves in much the same way as *Euglena gracilis*. The strains of the former, kept at three different places, namely Delft, Prague and Cambridge, have eventually also lost the ability to produce colourless cells.

(*d*) *The concept of leucophytes*

The case of *E. gracilis* also has some bearing on Lwoff's concept of leucophytes which, according to him, are characterized by biochemical and cytological features, namely by being oxytrophic and by possessing leucoplasts. 'Oxytrophic organisms are such as need an additional source of carbon whatever the source of nitrogen may be' (Lwoff, 1932, p. 113), and that can generally only be supplied in the form of an organic acid. Cytologically leucophytes are characterized by the possession of unpigmented plastids. Reserve substances like starch and paramylon are considered to be formed only by plastids, in the absence of photosynthesis from organic acids. According to this definition *Astasia longa*, *Chilomonas paramecium* and *Polytomella caeca* would not be true leucophytes because they multiply in media with beef extract, yeast extract or peptone without any addition of organic acids or other carbon source (Pringsheim, 1937, pp. 68–9; cf. also Lwoff, 1943, p. 106).

Lwoff has modified his definition several times, for instance also to elude his previous statement that only leucophytes are oxytrophic and that this mode of nutrition is not found in the presence of chlorophyll, a case actually established in *Chlorogonium* (Pringsheim, 1934). He was also forced to include organic substances other than acids. The latest definition (Lwoff, 1943, pp. 102, 106) regards as oxytrophic organisms which either need an additional source of carbon or multiply abundantly only if it is supplied.

This alteration of the definition renders it so vague that it becomes almost unusable, first because mixtures of peptones with beef or yeast extract are quite good media for the flagellates mentioned above, secondly because the original idea has really been abandoned, every organism promoted in its growth by any nitrogen-free organic substance being now 'oxytrophic'.

Even if the concept of oxytrophism were restricted to organisms favoured by lower fatty acids according to Lwoff's original idea, so many forms would be included that the link between this nutritional speciality and Lwoff's leucophytes would have to be given up. An undisputable promotion of growth by acetates is found in many Volvocales and Chlorococcales which Lwoff would hesitate to call oxytrophic, as well as in a great number of fungi and bacteria. Striking examples are *Caryophanon latum* and *Lineola longa* which are specifically furthered by acetate (Pringsheim & Robinow, 1947). These do not fit definitely into Lwoff's system of nutrition. The concept of oxytrophism should therefore be abandoned, even in its latest, repeatedly modified form.

The same is true of Lwoff's cytological definition of leucophytes. They should, according to his latest statement (1943, p. 72), 'possèdent un ou plusieurs plastes et sont dépourvus de chlorophylle'. Such organisms have not yet been found and do not seem to exist. As shown by Hovasse in the present paper, *Euglena gracilis* forma *hyalina* has definitely lost its plastids, although it still forms paramylon. It possesses a protoplasmic structure strikingly similar to that which Lwoff and his collaborators regard as a leucoplast in colourless Volvocales—and would certainly regard as such in the colourless Euglenineae, had they investigated any of these. In all these cases Lwoff misinterprets the chondriome as a plastidome.

Having thus eliminated these hypotheses and definitions of Lwoff's, the way has been opened for further study of the manifold modes in which apochlorotic organisms originate in nature.

VII. SUMMARY

Earlier observations have shown that clone cultures of *Euglena gracilis* occasionally produce colourless cells, from which completely colourless subclones can be reared. Two further instances are described in this paper.

Contrary to previous statements, reduction in the number of chromatophores plays only a secondary role in the genesis of apochlorotic individuals. The loss of chlorophyll and accessory pigments is due to disorganization of the plastid stroma, while the cells remain capable of multiplication.

The circumstances leading to plastid disorganization are at present unpredictable. The strains exhibiting this phenomenon showed an unstable period of plastid degeneration only once in the course of these investigations.

No special factor inducing this phenomenon could be found, and the majority of the strains of *E. gracilis* never produced apoplastidic cells. It is therefore likely that gene-mutation will be the main factor in their production.

E. gracilis multiplies abundantly in the dark in acetate-peptone solutions, especially if supplemented with beef or yeast extract. The strains originating from apoplastidic cells thrive just as well in such media and do not show any signs of being less vigorous than the green ones.

The apoplastidic clones derived from the two green strains under laboratory conditions are identical both with one another and with *Astasia longa* Pringsh., a species found on several occasions in nature. No morphological, cytological or physiological differences can be found.

The cytological investigations carried out by R. Hovasse show that neither the apochlorotic strains of *Euglena gracilis*, nor those of *Astasia longa* have any plastids left. The chondriome is of the same type in both, though less developed in the green strains. In these it exhibits no connexion with the plastidome. The colourless strains not only show no leucoplasts but also no trace of the eye-spot and of the flagellar thickening which is regarded as the light-sensitive organ. While the green cells are phototactic, the colourless ones are not.

The cytological, ecological and taxonomic implications of this instance of apoplastidy are discussed.

I want to express my indebtedness to those who provided me with material and to Prof. F. E. Fritsch for his advice and his help in correcting the text.

REFERENCES

ALEXANDER, G. (1931). The significance of hydrogenation concentration in the biology of *Euglena gracilis* Klebs. *Biol. Bull. Woods Hole*, **61**, 165.

BAKER, C. L. (1933). Studies on the cytoplasmatic compounds of *Euglena gracilis* Klebs. *Arch. Protistenk.* **80**, 434.

BAKER, W. B. (1926). Studies on the life history of *Euglena*. I. *Euglena agilis* Carter. *Biol. Bull. Woods Hole*, **51**, 321.

BEIJERINCK, M. W. (1904). *Chlorella variegata*, ein bunter Mikrobe. *Rec. trav. bot. néerl.* **1**, 14.

BENECKE, W. (1900). Ueber farblose Diatomeen der Kieler Föhrde. *Jb. wiss. Bot.* **35**, 535.

Biecheler, B. (1934). Mise en évidence d'un réseau mitochondriale chez quelques Péridiniens autotrophes. *C.R. Acad. Sci., Paris,* **199**, 1241.

Bolte, E. (1920). Ueber die Wirkung von Licht und Kohlensäure auf die Beweglichkeit grüner und farbloser Schwärmzellen. *Jb. wiss. Bot.* **59**, 287.

Brown, V. E. (1930). Cytoplasmic inclusions of *Euglena gracilis* Klebs. *Z. Zellforsch.* **11**, 244.

Calkins, G. N. (1933). *The Biology of the Protozoa.* Philadelphia.

Carter, H. J. (1856). Notes on the fresh-water Infusoria of the island of Bombay. I. Organization. *Ann. Mag. Nat. Hist.* ser. II, **18**, 240.

Causey, D. (1926). Mitochondria in *Euglena gracilis* Klebs. *Univ. Calif. Publ. Zool.* **28**, 217.

Chadefaud, M. (1935). Le cytoplasme des algues vertes. *Rev. algol.* **8**, .

Chadefaud, M. (1937). Recherches sur l'anatomie comparée des Eugléniens. *Botaniste,* **28**, 85.

Chadefaud, M. (1944). Les mitochondries des Euglènes. *Bull. Soc. bot. Fr.* **91**, 174.

Chatton, E. (1927). La microinclusion à l'agar-paraffine, technique protistologique et embryologique. *Bull. Histol. Tech. micr.* **4**, 1; *Bull. Soc. fr. micr.* **5**.

Dangeard, A. P. (1901). Recherches sur les Eugléniens. *Botaniste,* **8**, 97.

Doflein, H. (1929). *Lehrbuch der Protozoenkunde,* 5. Auflage. Jena: Neubearbeitet von E. Reichenow.

Dusi, H. (1930). La nutrition autotrophe d'*Euglena gracilis* Klebs aux dépens de quelques corps azotés inorganiques. *C.R. Soc. Biol., Paris,* **104**, 662.

Dusi, H. (1933). Recherches sur la nutrition de quelques Euglènes. *Ann. Inst. Pasteur,* **1**, 550, 840.

Dusi, H. (1937). Le besoin de substances organiques de quelques Eugléniens à chlorophylle. *Arch. Protistenk,* **89**, 94.

Elmore-Sauer, M. E. (1928). Antigenic properties of *Euglena gracilis. J. Immunol.* **15**, 21.

Elmore-Sauer, M. E. (1935). Correlation of immunologic and physiologic types of *Euglena gracilis* Klebs. *Arch. Protistenk.* **85**, 412.

Emerson, R. (1929*a*). Chlorophyll content and rate of photosynthesis. *Proc. Nat. Acad. Sci., Wash.,* **15**, 281.

Emerson, R. (1929*b*). The relation between maximum rate of photosynthesis and concentration of chlorophyll. *J. Gen. Physiol.* **12**, 600.

Frank, T. (1904). Kultur und chemische Reizerscheinungen der *Chlamydomonas tingens. Bot. Z.* **62**, 153.

Fritsch, F. E. (1935). *The Structure and Reproduction of the Algae.* Cambridge.

Guilliermond, A. (1934). Les constituants morphologiques du cytoplasme: le chondriome. *Act. Sci. Ind., Paris.*

Hall, R. P. (1939*a*). The question of autotrophic nutrition in *Euglena gracilis. Physiol. Zoöl.* **12**, 76.

Hall, R. P. (1939*b*). The trophic nature of the plant-like flagellates. *Quart. Rev. Biol.* **14**, 1.

Hall, R. P. & Jahn, T. L. (1929). On the comparative cytology of certain euglenoid flagellates and the systematic position of the families Euglenidae Stein and Astasiidae Bütschli. *Trans. Amer. Micr. Soc.* **48**, 388.

Hall, R. P. & Schoenborn, H. W. (1939). The question of autotrophic nutrition in *Euglena gracilis. Physiol. Zoöl.* **12**, 76.

Hansgirg, A. (1886–8). Prodromus der Algenflora von Böhmen. *Arch. naturw. LandesForsch. Böhm.* **5**, no. 6.

Hollande, A. (1938). Les dictyosomes des Eugléniens. *C.R. Soc. Biol., Paris,* **127**, 527.

Hollande, A. (1942). Étude cytologique et biologique de quelques Flagellés libres. *Arch. Zool. exp. gén.* **83**, 1.

Hovasse, R. (1923). *Endodinium chattoni,* parasite des vélelles. *Bull. biol.* **57**, 308.

Hovasse, R. (1937). Aux confins du règne animal: les constituants cytoplasmiques des Volvocales. *Bull. Soc. zool. Fr.* **62**, 107.

Hovasse, R. (1938). Nouvelles recherches sur les constituants cytoplasmiques des Volvocales. *Bull. Soc. zool. Fr.* **63**, 357.

Jahn, T. L. (1931). The effects of hydrogen-ion concentration on the growth of *Euglena gracilis* Klebs. *Biol. Bull. Woods Hole,* **61**, 387.

Jahn, T. L. & Kibben, W. R. Mc. (1937). A colourless euglenoid flagellate *Khawkinea halli,* nov. gen., nov. spec. *Trans. Amer. Micr. Soc.* **56**, 48.

Klebs, G. (1883). Ueber die Organisation einiger Flagellaten-Gruppen und ihre Beziehungen zu Algen und Infusorien. *Unters. Bot. Inst. Tübingen,* **1**, 233.

Klebs, G. (1893). Flagellatenstudien. *Z. wiss. Zool.* **55**, 353.

Kostir, W. J. (1921). The comparative resistance of different species of Euglenidae to citric acid. *Ohio J. Sci.* **21**, 267.

Lackey, J. B. (1934). Studies in the life history of Euglenidae. IV. A comparison of the structure and division of *Distigma proteus* Ehr. and *Astasia Dangeardi* Lemm. *Biol. Bull. Woods Hole,* **47**, 145.

Lackey, J. B. (1940). Limitations of Euglenidae as polluted water indicators. *Publ. Hlth Rep., Wash.,* **55**, 268.

Lichtenstein, S. (1922). Agglutination bei Algen, Hefen und Flagellaten. *S.B. preuss. Akad. Wiss.,* Phys. math. Kl., **127**.

LINSBAUER, K. (1915). Notiz über die Säureempfindlichkeit der Euglenen. *Öst. bot. Z.* **65**, 12.

LWOFF, A. (1932). *Recherches biochimiques sur la nutrition des Protozoaires.* Paris.

LWOFF, A. (1943). *L'évolution biologique.* Paris.

LWOFF, A. & DUSI, H. (1929). Le pouvoir de synthèse d'*Euglena gracilis* cultivée à l'obscurité. *C.R. Soc. Biol., Paris,* **102**, 567.

LWOFF, A. & DUSI, H. (1931). La nutrition carbonée et azotée d'*Euglena gracilis* en culture pure à l'obscurité. *C.R. Soc. Biol., Paris,* **107**, 1068.

LWOFF, A. & DUSI, H. (1935). La suppression expérimentale des chloroplastes chez *Euglena mesnili. C.R. Soc. Biol., Paris,* **119**, 1092.

LWOFF, A. & PROVASOLI, L. (1935). La nutrition de *Polytoma caudatum* var. *astigmatum* et la synthèse de l'amidon par les leucophytes. *C.R. Soc. Biol., Paris,* **119**, 90.

MAINX, F. (1927). Beiträge zur Morphologie und Physiologie der Eugleninen. I, II. *Arch. Protistenk.* **60**, 305, 355.

MAST, S. O. (1938). Factors involved in the process of orientation of lower organisms in light. *Biol. Rev.* **13**, 186.

MEYER, H. (1933). Das Chlorose- und Panaschüre-Problem bei *Chlorella. Beih. Bot. Zbl.* II, **51**, 170.

MOROFF, T. (1904). Beitrag zur Kenntnis einiger Flagellaten. *Arch. Protistenk.* **3**, 69.

PASCHER, A. (1916). Zur Auffassung der farblosen Flagellatenreihen. *Ber. dtsch. bot. Ges.* **34**, 440.

PASCHER, A. (1917). Flagellaten und Rhizopoden in ihren gegenseitigen Beziehungen. *Arch. Protistenk.* **38**, 1.

PASCHER, A. (1927). *Süsswasserflora: Volvocales,* p. 34. Jena.

PRINGSHEIM, E. G. (1908). Ueber die Herstellung von Gelbfiltern und ihre Verwendung zu Versuchen mit lichtreizbaren Organismen. *Ber. dtsch. bot. Ges.* **26a**, 556.

PRINGSHEIM, E. G. (1912). Kulturversuche mit chlorophyllführenden Mikroorganismen. II. Zur Physiologie der *Euglena gracilis. Beitr. Biol. Pfl.* **12**, 1.

PRINGSHEIM, E. G. (1921). Zur Physiologie der saprophytischen Flagellaten. *Beitr. allg. Bot.* **2**, 88.

PRINGSHEIM, E. G. (1926). Ueber das Ca-Bedürfnis einiger Algen. *Planta,* **2**, 555.

PRINGSHEIM, E. G. (1934). Ueber Oxytrophie bei *Chlorogonium. Planta,* **22**, 146.

PRINGSHEIM, E. G. (1936). Zur Kenntnis saprotropher Algen und Flagellaten. I. Ueber Anhäufungskulturen polysaprober Flagellaten. *Arch. Protistenk.* **87**, 43.

PRINGSHEIM, E. G. (1937). Die Stellung der Azetatflagellaten in einem physiologischen Ernährungssystem. *Planta,* **27**, 61.

PRINGSHEIM, E. G. (1942). Contributions to our knowledge of saprophytic algae and flagellata. III. *Astasia, Distigma, Menoidium* and *Rhabdomonas. New Phytol.* **41**, 171.

PRINGSHEIM, E. G. (1946*a*). *Pure Cultures of Algae.* Cambridge.

PRINGSHEIM, E. G. (1946*b*). The biphasic or soil-water culture method for growing Algae and Flagellata. *J. Ecol.* **33**, 193.

PRINGSHEIM, E. G. & ROBINOW, C. F. (1947). Observations on two very large Bacteria, *Caryophanon latum* Peshkoff and *Lineola longa* (nomen provisorium). *J. Gen. Microb.* **1**, 267.

PROVASOLI, L. (1937-8). Studi sulla nutrizione dei Protozoi. *Boll. Zool. agrar. bach.* **8**, 1.

RABINOVICH, D. (1940). Existencia de un leucoplasto en polytomella caeca.... *Rev. Inst. Bact.* no. 4, p. 424.

REICHENOW, E. (1909). Untersuchungen an *Haematococcus pluvialis* nebst Bemerkungen über andere Flagellaten. *Arb. GesundhAmt.* **33**, 1.

ROTHERT, W. (1914). Der 'Augenfleck' der Algen und Flagellaten—ein Chromoplast. *Ber dtsch. bot. Ges.* **32**, 91.

SCHERFFEL, A. (1911). Beitrag zur Kenntnis der Chrysomonadinen. *Arch. Protistenk.* **22**, 301.

SCHMITZ, FR. (1884). Beiträge zur Kenntnis der Chromatophoren. *Jb. wiss. Bot.* **15**, 1.

SCHOENBORN, H. W. (1940). Studies on the nutrition of colorless euglenoid flagellates. 1. Utilization of inorganic nitrogen by *Astasia* in pure culture. *Ann. New York Acad. Sci.* **40**, 1.

SCHOENBORN, H. W. (1942). Studies on the nutritional requirements of *Euglena gracilis* in darkness. *Physiol. Zoöl.* **15**, 325.

SMITH, G. M. (1933). *The Freshwater Algae of the United States.* New York.

TANZER, C. (1941). Serological studies with free-living *Protista. J. Immunol.* **42**, 291.

TERNETZ, C. (1912). Beiträge zur Morphologie und Physiologie der *Euglena gracilis* Klebs. *Jb. wiss. Bot.* **51**, 435.

VOLKONSKY, M. (1930). Les constituants cytoplasmiques de *Polytoma uvella* Ehrbg. Existence d'un leucoplaste. *C.R. Soc. Biol., Paris,* **105**, 619.

ZUMSTEIN, H. (1900). Zur Morphologie und Physiologie der *Euglena gracilis* Klebs. *Jb. wiss. Bot.* **34**, 149.

THE RECENT MIGRATION OF CERTAIN INDO-PACIFIC ALGAE FROM THE RED SEA INTO THE MEDITERRANEAN

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(With Plates 1 and 2 and 1 figure in the text)

Our knowledge of the marine algae of the Eastern Mediterranean is still scanty, although the western shores have been more fully investigated by algologists such as Falkenberg (1901), Funk (1927), Hamel (1931*a*), and Feldmann (1931*a*, *b*, 1937, 1943). During a study of the algal vegetation on the shores of the Eastern Mediterranean, particularly of those of Egypt, the writer has found a number of species which appear to be new records. The species in question are for the most part known to occur in the Indian Ocean, and their discovery in the Mediterranean is of interest in relation to some problems of geographical distribution.

NOTES ON THE SPECIES

(1) *Cladophoropsis Zollingeri* (Kütz.) Boergesen, 1933, p. 1, fig. 1

This species forms deep green tufts, 2–3 cm. high, attached to rocks by rhizoids and tenaculae. It occurs, particularly during summer, in the *Acanthophora*-community both in the upper and lower littoral belts* and in exposed and sheltered situations alike.

The ramification of the filaments (Pl. 1, fig. 1) is irregular, the lateral branches being either secund and then sometimes decumbent or very rarely subopposite. The apices of the branches are obtuse. The distance between the septa varies widely and the breadth of the filaments is 140–200μ in agreement with Boergesen.

Locality. Widespread near Alexandria and Sallum.

Geographical distribution. Indian Ocean (Red Sea, Arabian Sea, India, Ceylon), Indo-pacific region, Pacific Ocean (Japan, China), Cape Horn region.

(2) *Acetabularia Moebii* Solms-Laubach, 1895, p. 30, pl. 4, fig. 1

The entire plant (Pl. 1, fig. 2) measures only a few millimetres, generally 5–8. It was found in the *Dilophus*-community just below low-water mark, attached to small bivalved shells hidden by other algae. It was also dredged from deep water near Alexandria.

The sporangial rays are 14–17 in number and are without an inferior corona. There are 4–6 hair scars on the superior corona.

Locality. Alexandria (East Harbour, Sidi Bishr).

Geographical distribution. Indian Ocean (Red Sea, India, Ceylon), Pacific Ocean (Japan, China).

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* The amplitude of the tides in the Mediterranean is small.

(3) *Gracilaria arcuata* Zanardini, 1858, p. 265, pl. 5, fig. 2; Okamura 1929, p. 40, pl. 272, figs. 1–6; Feldmann, 1931*b*, p. 14, figs. 4–6

The specimens agree fairly well with Feldmann's description and figures. The tufts were 10 cm. high; the branches and ramulae being thick, rounded, unilaterally disposed and incurved. Cystocarps occur in autumn.

Locality. Alexandria, Port Said and Port Fouad.

Geographical distribution. Atlantic Ocean (West Indies), Mediterranean Sea, Indian Ocean (Red Sea, India, Ceylon), Indo-pacific region, Pacific Ocean (Japan, China, Australia, Polynesia).

(4) *Sarconema furcellatum* Zanardini, 1858, p. 50, pl. 8, fig. 1; Kylin, 1932, p. 21; Boergesen, 1939, p. 111

Boergesen, after examining material from the Indian Ocean and the Red Sea, came to the conclusion that three out of the four species of *Sarconema* enumerated by Kylin are only habitat forms of Zanardini's plant. Even the fourth species (*S. filiforme* (Sond.) Kylin) has not yet been sharply defined.

The Mediterranean specimens (Pl. 1, fig. 3) agree fairly well with *S. furcellatum*, as described by Zanardini from the Red Sea. Tetraspores occur in October.

Locality. Port Said and Port Fouad.

Geographical distribution. Indian Ocean (Red Sea, Arabian Sea, India, Ceylon).

(5) *Hypnea cornuta* (Lamour.) J. Ag. in Boergesen, 1920, p. 382; Taylor, 1928, p. 156, pl. 22, fig. 12; Tanaka, 1941, p. 242, fig. 14

The Mediterranean plants (Pl. 1, fig. 4) agree well with Boergesen's description, except for the absence of a thick cuticle. In this respect they resemble specimens found by Nasr in the Red Sea. The alga was found attached to rocks or intermingled with other algae in sheltered places in the Harbour.

Locality. Alexandria (East Harbour, Abu Qir Bay).

Geographical distribution. Atlantic Ocean (West Indies, Florida, Canary Islands), Indian Ocean (Red Sea), Indo-pacific region, Pacific Ocean (Japan, China).

(6) *Rhodymenia erythraea* Zanardini, 1858, p. 276

So far no illustrated account has been given of this species. Zanardini, when he first described the plant from Yemen, published no figures with his description, and since then the species has not been recorded except among a collection from Eritrea made by Piccone (1884). As a consequence some authors have regarded it as a doubtful species. In the Herbarium of the British Museum (Nat. Hist.) there is a sterile specimen collected by J. Murray from Karachi (Herb. Dickie, 1884) under the name *R. erythraea* Zanardini, but judging from the structure of the plant, it looks more like a *Calliblepharis*.

The fronds arise from a small disk, are distinctly cuneate at the base and subcoriacious in texture; they are branched in a palmate manner, often dichotomously (Pl. 2). The basal segments have a smooth margin, while the upper ones sometimes bear numerous marginal proliferations giving the frond a denticulate appearance. The apical segments are much divided and bear more or less obtuse ligulate proliferations. The plant does not adhere well to paper.

Transverse sections of the thallus show a medulla of two to three layers of large oblong cells, and a 'nemathecioid' cortex which is well developed only in tetrasporic individuals.

The thickness of the thallus is 250–300μ. The tetrasporangia (Pl. 1, fig. 5) are superficial and scattered over the whole thallus; they are cruciately divided. The procarp (Pl. 1, fig. 7) consists of a large multinucleate basal cell, an auxiliary mother cell, an auxiliary cell and a 3-celled carpogonial branch. There are few cystocarps in the basal segments, but they are densely aggregated above. They are present on both surfaces and even occur on the marginal proliferations. They are hemispherical, sessile and protuberant (Pl. 1, fig. 6).

Healthy individuals reach a length of 10–12 cm. and are rose to yellowish red in colour. The tetrasporic plants are not as broad as the sexual plants but are more divided than the latter.

The general appearance of the thallus recalls that of *Rhodymenia palmata*, as pointed out by Zanardini, but differs from the latter in texture, size, colour, the denticulate character of the frond and the less developed cortex; cystocarps, moreover, have not yet been discovered in *R. palmata*.

There has been some confusion as to the systematic position of the species. In his revision of the genus *Rhodymenia*, Dawson (1941) suggested the creation of a new section '*Pertusae*', distinguished from Agardh's section '*Palmatae*' by the presence of 'an essentially unmodified cortex in *Pertusae*' as compared with 'a nemathecially modified cortex in *Palmatae*' (p. 129). This distinction does not seem to be a sharp one, because even in one and the same plant the structure of the cortex may differ in different parts of the thallus, especially as between tetrasporic and sexual plants of the same species. Dawson instituted the section *Pertusae* after examining tetrasporic material of only one of the seven species which are included in this new section.

Although he assigned *Rhodymenia erythraea* to it, it seems that he was not very confident about this species which he described as 'of doubtful affinity' (p. 125), adding that 'more material from the Red Sea, may eventually establish this species as a definite entity'. *R. erythraea*, however, besides possessing a more or less modified 'nemathecioid' cortex, has the tetrasporangia scattered over the whole surface. It should therefore be included in the section *Palmatae* J.Ag. (p. 329).

The species was collected on the quays at Port Said Harbour at a depth of 0–50 cm. Its presence in the Mediterranean is possibly due to conveyance from the Indian Ocean on ships' bottoms.

Locality. Port Said Harbour.

Geographical distribution. Indian Ocean (Red Sea, African coast).

DISCUSSION

The discovery in the Eastern Mediterranean of these six conspicuous algae, most of which could not readily be overlooked, raises important questions as to the relations between the marine flora of the Mediterranean and that of the Red Sea. Analysis of the flora of the Mediterranean had previously revealed the presence of an Indo-pacific element (cf. Feldmann, 1937; Fritsch, 1945) and the problems relating to its origin have been discussed by various writers. A résumé of the different views put forward may be as follows:

(1) That invasion took place from the west (through Gibraltar), assuming that the Mediterranean is a part of the Atlantic which according to Murray (1893) might have received its Indo-pacific algae by way of migration via the Cape during hypothetical periods of tropical conditions at the southern extremity of Africa.

(2) That invasion took place from the south-east by one or both of the following ways: (*a*) by the open connexion between the Mediterranean and the Red Sea existing during the Tertiary period (Svedelius, 1924); (*b*) by way of the Suez Canal and after its construction (Forti, 1928).

Murray put forward his theory of climatic change before the flora of the Atlantic had been more fully explored by Boergesen. The theory was discussed by Svedelius (1924, p. 6) who showed that, although it might explain the distribution of the arctic element, it was improbable that it could apply to the tropical and subtropical elements. He advocates utilizing the interoceanic connexions during the Tertiary to explain the discontinuous distribution of certain tropical algae and concluded that the flora of the Indo-pacific Ocean is perhaps more ancient than that of the Atlantic (p. 61).

The possible part played by the Tertiary connexion between the Mediterranean and the Red Sea has been discussed by various writers. Ascherson (1871) was one of the first to draw attention to it in relation to the distribution of the sea-grasses *Posidonia* and *Cymodocea* (cf. also Ostenfeld, 1918). Hamel (1931*b*) also supported the theory of a migration in Tertiary times on the grounds that certain Indian algae (e.g. *Liagora farinosa*) were known to occur in the Mediterranean before the establishment of the Suez Canal.

It is, however, well to be cautious about drawing a definite conclusion as to the validity of this theory, at least with regard to the algae, for the following reasons:

(1) The Indo-pacific element is still poorly represented in the Mediterranean.

(2) The number of species newly recorded from the Mediterranean exceeds that known prior to the opening of the Suez Canal.

(3) Our knowledge of the geographical distribution of the species of marine algae is still in its infancy. Species which were once considered to be Indo-pacific seem to have a world-wide distribution in warmer seas.

(4) The 'vicarious' species, which Svedelius cites in support of his theory, are met with also in other seas which are not separated by continents. For example, *Rhodymenia palmata* and *R. palmatiformis* are two very nearly related species found in the northern and southern hemispheres respectively, although lacking in the equatorial region.

(5) There are numerous examples of other algae with a discontinuous distribution which cannot be explained as due to former interoceanic connexions. One instance is furnished by *Antithamnion Lherminieri*, which was first described by Crouan from Guadeloupe and recently recorded by Nasr (1939) from the Red Sea, although it is unknown in the intervening Mediterranean region. For other examples cf. Fritsch (1945, p. 12).

(6) In reviewing the species recorded from the Mediterranean and cited by the different authors in favour of the theory, one can safely say that not a single example of the Indo-pacific phaeophyceae is represented. The bulk of chlorophyceae and rhodophyceae are represented by genera whose species are either equally distributed between the Mediterranean and the Atlantic, or broadly described as pantropical, i.e. known from all warmer seas, such as: *Liagora*, *Vidalia*, *Codium*, *Scinaia* and *Dasycladus*. Of these examples, *Dasycladus claeviformis* had its origin in the Mediterranean, *Scinaia furcellatum* is described by Svedelius (p. 61) as having a very uncertain path of immigration into the Mediterranean. *Scinaia complanata*, on the other hand, has no doubt entered the Mediterranean from the west. It is known from the Atlantic, was recorded by Feldmann (1937) from Banyuls and was found by the writer near Alexandria. *Acanthophora Delilei*

is perhaps the only example in favour of the theory. Once it showed a remarkable discontinuity between the Indian Ocean and the Mediterranean (although the western shores of Africa are still poorly explored).

There is another possibility, which has not previously been mentioned, to account for the presence in the Mediterranean of species from the Indian Ocean such as *Acanthophora Delilei* as well as of the sea-grasses mentioned above. The migration could have taken place in historical times prior to the opening of the Suez Canal and after the termination of the Tertiary period, through the old intermittent canal which was established by the Ancient Egyptians between the Mediterranean and the Red Sea via the Wadi Tomilat depression. Evidence from the manuscripts recently discovered in the temples, has shown that this canal was used for navigation between the two seas by different successive Dynasties (Victor, 1934; and others).

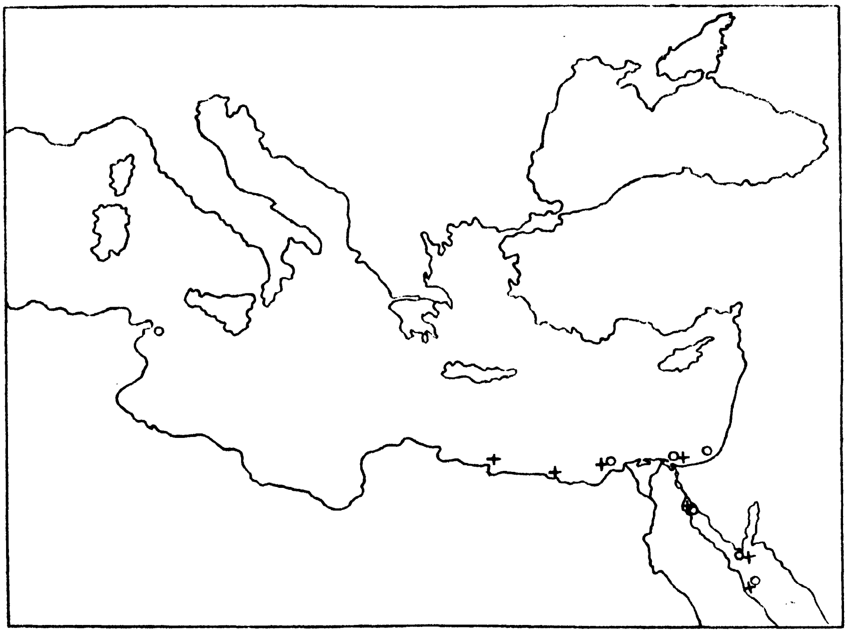
The present role of the Suez Canal as a means for the migration of marine fauna and flora between the two seas has become more evident. The appearance of the sea-grass *Halophila Stipulacea* near Rhodes (collected by K. Fritsch in 1894) was perhaps the first instance discovered that drew attention to the probable importance of the Suez Canal in this connexion. Later, in 1924, the same plant was found by the Cambridge Expedition in the Suez Canal watercourse. In 1898, Reinbold reported the presence of *Hypnea valentia* and *H. nidifica* at Rhodes and suggested their immigration from the Red Sea through the newly cut canal. Forti (1928) attributed the presence of *Hypnea musciformis*, *H. nidifica* and *Hydroclathus cancellatus* near the Dodecanese to their passage as epiphytes on other vegetation through the Suez Canal. J. & G. Feldmann (1938) also reported the presence of *Lophocladia Lallemandi* in Algeria. Its apparent absence from the Atlantic is in favour of its migration from the Red Sea.

The new records given in this paper appear to the writer to lend further support to the theory of a migration through the Suez Canal. The species in question were not previously known to occur in the Mediterranean, except for *Gracilaria arcuata* which is recorded by Feldmann (1931*b*) from Tunis. This species seems to have a world-wide distribution in the warmer seas and could scarcely be described as Indo-pacific. This is equally true of *Hypnea cornuta*. Yet, it is probable that these two species have entered the Mediterranean from the south-east, where they are widely distributed. *Hypnea cornuta*, although recorded from the Canary Islands, seems to be absent from the Western Mediterranean and this suggests its migration through the Isthmus of Suez.

Cladophoropsis Zollingeri, a typical Indo-pacific member of chlorophyceae, is of interest owing to its westward migration in the Mediterranean, since it has established itself near Sallum, 500 km. to the west of Alexandria. Its distribution in the Mediterranean, together with that of *Gracilaria arcuata*, is shown in Text-fig. 1. The remaining species discussed in this paper may in course of time spread in the same way.

In spite of the fact that most of these species are known to occur in the northern part of the Red Sea (Nasr, 1940), as well as in the south-eastern part of the Mediterranean, they are lacking among the algae listed by Lyle (1930) from the waters of the Suez Canal. Subsequent workers have likewise been unsuccessful in finding them. The question therefore arises why these species have failed to establish themselves in the Canal. The writer is of the opinion that this may be due—at least during certain seasons of the year—to the wide range of salinity of the waters of the Suez Canal. According to Faouzi (1947) and Ghazzawi (1939) the salinity fluctuates between 30 and 48 $^0/_{00}$ and may even, during certain months of the year, become as low as 20°/$_{00}$ at a station on Manzala Lake near Port Said, while reaching only a slightly higher value at Ismailia. These low salinity values are mainly due to the discharge of flood waters through Lake Manzala and through Lake Timsah from the branches of the Nile.

Faouzi points out that, while Suez water reaches the Mediterranean, water from the latter never reaches the Red Sea, there being no direct current from Port Said to Suez. This may throw light on the relative preponderance of Red Sea species in the Mediterranean as compared with Mediterranean species in the Red Sea.



Text-fig. 1. Distribution of *Gracilaria arcuata* (o) and *Cladophoropsis Zollingeri* (+) in the Mediterranean.

It may be concluded that the species discussed in this paper have succeeded in traversing the Canal at times when the salinity was favourable, being conveyed either on ships' bottoms or with the help of the Suez current.

SUMMARY

1. The following species are newly recorded in the Mediterranean flora: *Cladophoropsis Zollingeri* (Kütz.) Boergesen, *Acetabularia Moebii* Solms, *Sarconema furcellatum* Zanardini, *Hypnea cornuta* (Lamour.) J.Ag. and *Rhodymenia erythraea* Zanardini. They are mostly Indo-pacific in their distribution, and it is suggested that they have migrated into the Mediterranean via the Suez Canal.

2. The systematic position of *Rhodymenia erythraea* Zanardini is discussed.

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REFERENCES

AGARDH, J. G. (1876). *Species, genera et ordines Algarum*, **3** (Epicrisis system. Florid.). Lipsiae.

ASCHERSON, P. (1871). Die geographische Verbreitung der Seegräser. *Petermanns Mitt.* **17**, 241.

BOERGESEN, F. (1920). *The Marine Algae of the Danish West Indies*, **2**. Copenhagen.

BOERGESEN, F. (1933). Some Indian green and brown algae especially from the shores of the presidency of Bombay. *J. Indian Bot. Soc.* **12**, 1.

BOERGESEN, F. (1930). Marine algae from the Iranian Gulf. *Danish Scientific Investigations in Iran*, **1**, 47. Copenhagen.

DAWSON, E. Y. (1941). A review of the genus *Rhodymenia*. *Allan Hancock Pacific Expeditions*, **3**, nos. 7, 8. California.

FALKENBERG, P. (1901). Die Rhodomelaceen des Golfes von Neapel. *Fauna u. Flora Neapel*, **25**.

FAOUZI, H. (1947). *Régime des courants dans le Canal de Suez* (in the Press).

FELDMANN, J. (1931*a*). Contribution à la flore alg. mar. de l'Algérie. *Bull. Soc. Hist. nat. Afr. N.* **22**, 179.

FELDMANN, J. (1931*b*). Note sur quelques alg. mar. de Tunisie. *St. Ocean. de Salambô, Notes*, no. 24. Tunisie.

FELDMANN, J. (1937). Recherches sur la vegetation marine de la Mediterranée (côte des Alberes). *Rev. algol.* **10**, 1–339.

FELDMANN, J. & G. (1938). Présence du *Lophocladia Lallemandi* (Mont.) Schmitz. aux environs d'Alger. *Bull. Soc. Hist. nat. Afr. N.* **29**, 479.

FELDMANN, J. & G. (1942). Additions à la flore des alg. mar. de l'Algérie. *Bull. Soc. Hist. nat. Afr. N.* **33**, 230.

FORTI, A. (1928). Nuove segnalazioni di Alghe passivamente transportata a traverso al canal di Suez. *Nuov. G. bot. ital.*, N.S., **34**, 1443.

FRITSCH, F. E. (1945). *The Structure and Reproduction of the Algae*, **2**. Cambridge.

FUNK, G. (1927). Die Algenvegetation des Golfes von Neapel. *Pubbl. Staz. zool. Napoli*, Suppl. **7**.

GHAZZAWI, F. (1939). A study of the Suez Canal plankton. *Hydr. and Fisher. Directorate, Notes et Memoirs*, no. 24. Cairo.

HAMEL, G. (1931*a*). Chlorophycée des côtes françaises. *Ex. Rev. Alg.* 1–5. Paris.

HAMEL, G. (1931*b*). Sur la *Cladostephus dubius Bory*. *Trav. crypt. dédiés a L. Mangin*, p. 309. Paris.

KYLIN, H. (1932). Die Floridenordnung Gigartinales. *Lunds Univ. Arrsk.*, N.F., **28**, no. 8.

LYLE, L. (1930). Algae of the Suez Canal. *J. Bot., Lond.*, **68**, 327.

MURRAY, G. (1893). A comparison of the marine floras of the warm Atlantic, Indian Ocean and the Cape. *Phyc. Memoirs*, **2**, 65. London.

NASR, A. H. (1939). Some new and little known algae from the Red Sea. *Rev. algol.* **11**, 10.

NASR, A. H. (1940). Chorography of the marine algae inhabiting the northern part of the Red Sea. *Bull. Inst. égypt.* **22**, 193.

OKAMURA, K. (1929). *Icones of Japanese Algae*, **6**, no. 5. Tokyo.

OSTENFELD, C. H. (1918). Sea grasses. *Report on the Danish Ocean. Exped.* (1908–1910) *to the Mediterranean and Adjacent Seas*, **2**, K. 2. Copenhagen.

PICCONE, A. (1884). Contribuzioni all 'Algologia eritrea. *Nuov. G. bot. ital.* **16**, 328.

REINBOLD, T. (1898). Meeresalgen von der Insel Rhodes. *Hedwigia*, **37**, 87.

SOLMS-LAUBACH (1895). Monograph of the Acetabulariae. *Trans. Linn. Soc., Lond.*, **5**, pt. 1.

SVEDELIUS, N. (1924). On the discontinuous geographical distribution of some tropical and subtropical marine algae. *Ark. Bot.* **19**, no. 3.

TANAKA, T. (1941). The genus *Hypnea* from Japan. *Inst. Algol. Res.* **2**, no. 2.

TAYLOR, W. R. (1928). Marine algae of Florida. *Publ. Carnegie Instn*, no. 379.

VICTOR, ST. (1934). *Le Canal de Suez*. Paris.

ZANARDINI, G. (1858). Plantarum in mari rubro Hucusque collectarum. *Inst. Venet.* **7**, pt. 2.

EXPLANATION OF PLATES

PLATE 1

Fig. 1. *Cladophoropsis Zollingeri* (Kutz.) Boerg., part of thallus. (× 10.)

Fig. 2. *Acetabularia Moebii* Solms. (× 2.)

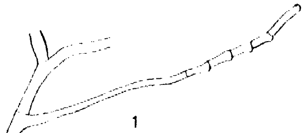
Fig. 3. *Hypnea cornuta* (Lamour.) J.Ag. ($\times \frac{3}{10}$.)

Fig. 4. *Sarconema furcellatum* Zanardini. ($\times \frac{4}{7}$.)

Figs. 5–7. *Rhodymenia erythraea* Zanardini. Fig. 5: transverse section of part of thallus showing tetrasporangia (× 143); fig. 6: cystocarp (× 100); fig. 7: procarp (× 630). (*a*, auxiliary cell; *am*, auxiliary mother cell; *b*, basal cell; *c*, carpogonial branch.)

PLATE 2

Rhodymenia erythraea Zanardini. (Nat. size.)



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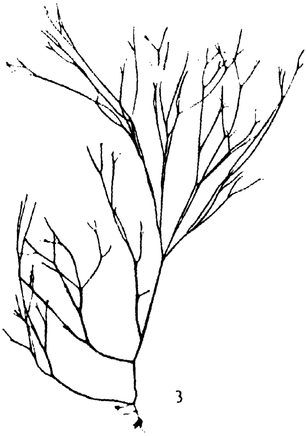

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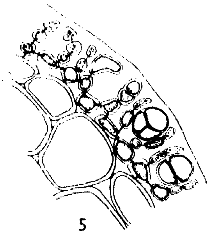

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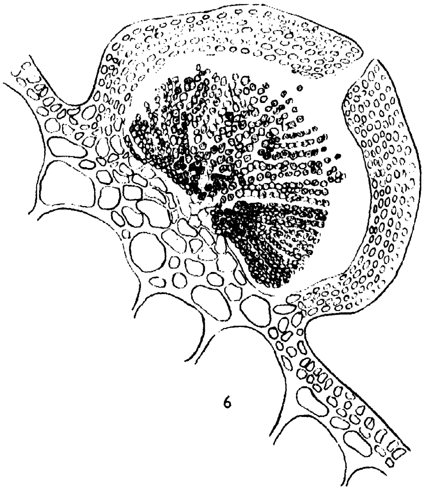

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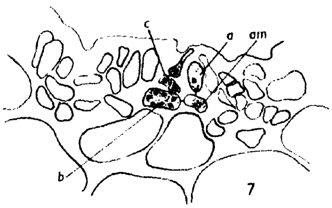

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ALEEM—THE RECENT MIGRATION OF CERTAIN INDO-PACIFIC ALGAE FROM THE RED SEA INTO THE MEDITERRANEAN

ALEEM—THE RECENT MIGRATION OF CERTAIN INDO-PACIFIC ALGAE FROM THE RED SEA INTO THE MEDITERRANEAN

THE NATURE AND RANGE OF VARIATION IN THE FLORAL SYMMETRY OF *POTENTILLA ERECTA* (L.) HAMPE

By A. G. TANSLEY

(With 35 figures in the text)

More than fifty years ago I read to the Linnean Society a paper on this subject, prepared in collaboration with Miss Elizabeth Dale. I was somewhat dissatisfied with it at the time because of the number of 'loose threads' involved in the complex problems of variation in which I was then interested, and I did not see how to follow them up; so the paper was never submitted for publication. In the middle nineties of the last century the subject of variation was very prominent in the minds of biologists. Bateson's thick volume, *Materials for the Study of Variation*, had recently been published, and controversy raged between those who thought that 'continuous' variation was the main, if not the only, material on which natural selection worked and those who stressed the importance of 'discontinuous' variation in evolution. It was, of course, before Johannsen's work on the non-inheritance of 'continuous' (fluctuating) variation, and before the rediscovery of Mendel's work and the revolution which it brought into this whole field.

My attention had been attracted by the constant occurrence of a small percentage of pentamerous flowers in the common tormentil, *Potentilla erecta* (then usually known as *Potentilla Tormentilla*), whose normal 4-petalled flowers are familiar to everyone, since the tormentil is one of the most widely distributed and commonest plants in rough grazings, heathlands and moorlands on the lighter non-calcareous soils throughout the country. Influenced by the notion that particular environmental conditions, such for instance as soil differences, water supply, local climate, or aspect, might affect this kind of variation Miss Dale and I collected and examined a large number of flowers from various sites in different parts of the country, but got no clear indications of any such effects. I did, however, conclude that the plant produces more pentamerous flowers at the outset of its flowering season than later in the summer. Thus I observed 10–12% of pentamerous flowers borne by luxuriant plants with long shoots which had grown up through gorse bushes and just opened their first flowers in June, at a time when the plants growing in grazed turf, and thus fully exposed to light from the first, had been flowering for some weeks. It is possible that the large proportion of pentamerous flowers (about four times the normal percentage) on these plants growing among gorse was connected with their luxuriance, but the correlation with early production is confirmed by the larger proportion of pentamerous flowers observable in early May, when fully exposed tormentils begin to flower, than later in the season. Further confirmation was obtained by observing the symmetry of successively opening flowers in pot cultures. Unfortunately, no systematic observations were made on the distribution on individual plants or on the seasonal distribution of variation in the symmetry of the flowers. It may be conjectured that pentamerous flowers are largely the terminal flowers of the main flowering axes, as in many other plants with normally tetramerous flowers, but this conjecture has not been verified.

The manuscript of the unpublished paper still exists, and it seems to me now to contain material of general interest in floral morphology: for this reason I am venturing to publish some of the data in the form of the present contribution.

VARIATION IN SYMMETRY OF THE COROLLA

Of 5078 flowers collected in large samples picked at random from very various sites and at various times in middle and late summer, 4925 had 4 petals, 124 had 5, 19 had 3, and 10 had 6, representing respectively 97, 2·44, 0·37 and 0·19% of the whole, and a total deviation from tetramery in the corolla of 3·0%. In the larger samples, each numbering 500 flowers or more, and including altogether 4728 flowers, the extreme limits of deviation from tetramery were 2·3 and 3·4%.

In 1833 C. C. Babington had published a paper intended to show that the Linnean genus *Tormentilla* should be merged in *Potentilla* (as has been done), because of the relative frequency of pentamery in the tormentil, the generic separation being based mainly on the tetramery of its typical flowers. Out of 2794 flowers Babington collected at the beginning of September he found larger percentages of pentamerous and hexamerous flowers than we did, but none which were trimerous. The differences between our percentage figures and Babington's remain unexplained, nor is speculation worth while, for neither his nor our collections were made in a way to satisfy modern statistical requirements. The value of the present paper must rest mainly on its records of the *types* of deviation from symmetry that actually occur. The quantitative data can do no more than indicate the *order* of the numbers in which they are found.

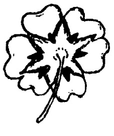
Table 1

	Tansley and Dale		Babington	
	Numbers and percentages		Numbers and percentages	
3 petals	19	0·37	—	—
4 petals	4925	97·00	2677	95·85
5 petals	124	2·44	110	3·9
6 petals	10	0·19	7	0·25
	5078	100·00	2794	100·00

Examples of flowers ranging from hexamerous to trimerous are represented, as seen from below, in Figs. 1–9, with intermediate forms apparently depending, as we shall see in the sequel, on gradually increasing curtailment of the circumference of the receptacle as we pass from one type of actinomorphic symmetry to the next below.

THE NORMAL TETRAMEROUS FLOWER

The tormentil flower is somewhat 'perigynous', i.e. the receptacle is considerably expanded and slightly concave above. The calyx consists of four ovate *sepals* appearing as outgrowths from the edge of the receptacle and with their broad bases in lateral contact. Alternating with the sepals and inserted just below the angles made by the sides of adjacent sepals are the four, usually linear or linear-lanceolate, members of the *epicalyx*. Immediately within these angles, i.e. on the upper surface of the receptacle, are inserted the four cuneate, obcordate or (rarely) orbicular *petals*, always with very narrow bases of attachment. The 16 *stamens* are in 3 whorls. The outermost whorl of 8 alternates with the eight members of the perianth, i.e. the stamens of this whorl are not opposite the centres of either petals or sepals, but each is midway between an antesepalous and the adjacent antepetalous radius. The 4 stamens of the next or middle whorl are antepetalous, and the 4 of the innermost whorl are antesepalous. The filaments of the antesepalous stamens are longer, those of the antepetalous stamens shorter, than those of the outer whorl. The *carpels* occupy the centre of the receptacle and may or may not fill up the whole space within the two inner whorls of stamens. They are very variable in number, ranging in our records from 2 to 15, but the range from 4 to 8 carpels is far the commonest. Out of 1171 normal tetramerous flowers with 16 stamens no less than 1076 fell within this range. Four carpels was the commonest number and there was a minor mode on the curve at 8 carpels (Graph I, p. 101). It will be noted that only the flowers the number of whose carpels falls on one of these modes (476 flowers out of 1171) are strictly *cyclic* throughout (eucyclic except for the double number of stamens in the outer whorl), following the formula Ak 4, K 4, C 4, A 8+4+4, G 4, or 4+4 (see Figs. 13, 14). The carpels of these cyclic flowers form a square in the centre of the flower: when there are 4 carpels these alternate with the antesepalous stamens, when there are 8 there is an additional inner whorl alternating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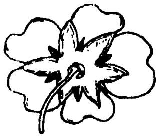
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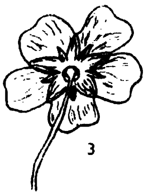
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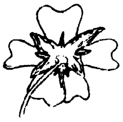
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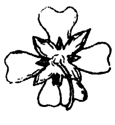
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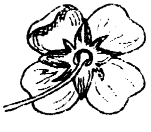
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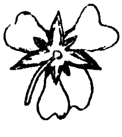
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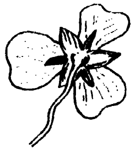
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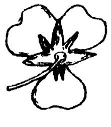
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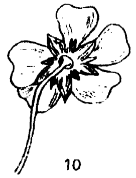
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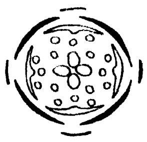
Figs. 1–9. Flowers ranging from hexamerous to trimerous, seen from below. Fig. 1. Flower with symmetrical hexamerous perianth. Fig. 2. Flower hexamerous in calyx and epicalyx, pentamerous in corolla (cf. Fig. 27). Fig. 3. Symmetrical pentamerous flower. Fig. 4. Pentamerous zygomorphic flower, one sepal and adjacent members of the epicalyx reduced, two petals overlapping. Fig. 5. As in Fig. 4 but sepal wanting. Fig. 6. Symmetrical tetramerous flower. Fig. 7. Tetramerous zygomorphic flower, one sepal and adjacent members of the epicalyx reduced, two petals overlapping. Fig. 8. Symmetrical trimerous flower. Fig. 9. Trimerous flower with zygomorphic perianth.

Fig. 10. Pentamerous flower with a member of the epicalyx bifid.

Fig. 11, 12. Double stamens: two intermediate stages of replacement of two adjacent stamens by a single one.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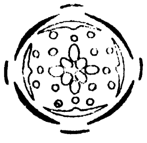
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Figs. 13 and 14. The normal tetramerous flower.

with the outer one. When the number of carpels is other than 4 or 8 but close to one of these numbers their placing often suggests a slight disturbance of the cyclic arrangement such as the development of one or two extra carpels or the failure of one or two to appear. But when the numbers are considerably higher than 8 there is a mass of carpels in the centre of the flower with no clear trace of cyclic arrangement.

VARIATION IN THE DIFFERENT WHORLS

The epicalyx

It is well known that the members of the epicalyx of the tormentil frequently show a tendency to 'doubling'. This may range from a notch at the apex of the single member (Fig. 10) to the appearance of two complete members side by side (Figs. 5 and 9). Out of a set of 1193 flowers whose perianths were fully recorded, 57 or 4·7% showed this tendency to doubling independently of differences in the symmetry of the flower as a whole. Of the 2794 flowers examined by Babington, 127 or 4·5% (a proportion very close to ours) showed this same tendency. No other whorl shows anything approaching the extent of this tendency to independent variation, and there can be little doubt of the correctness of the view of Payer (1857), earlier propounded by Auguste Saint-Hilaire on comparative grounds, that each member of a normal epicalyx represents two 'fused' stipules, one belonging to each of the adjacent sepals, the strong tendency to doubling representing 'reversion' to the primitive separateness of the two members, or, more strictly, failure to 'fuse', or to fuse completely.*

The calyx

The calyx of the tormentil is the most stable of the floral whorls. Independent variation of the number of sepals was only observed by us in 2 flowers (0·16%) and by Babington in 1 (0·03%).

The corolla

Independent variation of the number of petals is somewhat greater, but still very low. We found it in 7 (0·58%), Babington in 4 flowers (0·14%).

The andrœcium

Table 2 shows the variation in the number of stamens in 1176 out of a set of 1193 flowers.† In the remaining 17 it was impossible, from one cause or another, to count the stamens accurately.

Table 2

No. of stamens...	9	10	11	12	13	14	15	16	17	18	19	20	Totals	%
3 petals	—	2	—	6	2	1	—	—	—	—	—	—	11	0·93
4 petals	3	4	3	9	16	40	113	911	17	1	1	—	1118	95·07
5 petals	—	—	1	1	—	2	3	9	4	18	3	5	46	3·90
6 petals	—	—	—	—	—	—	—	—	1	—	—	—	1	0·08
Totals	3	6	4	16	18	43	116	920	22	19	4	5	1176	99·98

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* 'Fusion', in this connexion, of course means nothing else than the development of one organ in the place of two adjacent ones.

† This 'set' was not a 'sample' collected in one place, but includes flowers from various samples whose stamens were counted. The percentages of flowers deviating from tetramerous symmetry are different from those shown in Table 1.

It will be seen that the modes of the curves for the flowers with 3 and the flowers with 4 petals correspond with the typical floral formula, being at 12 and 16 stamens respectively, i.e. at 4*n*, where *n* is the number of petals, while that for the 5-petalled flowers is 18, not 20, with minor modes at 16 and 20. In both 4- and 5-petalled flowers there are far more flowers with a smaller number than with a larger number than the modal.

The positions of the stamens involved in the deviations from the number corresponding with the typical floral formula were recorded in 131 tetramerous flowers.* Of 10 flowers with 17 stamens 6 had an antepetalous stamen replaced by two close together, 3 had an antesepalous stamen similarly replaced, and in the remaining flower the extra stamen was situated midway between an antepetalous and an antesepalous stamen.

Of 73 tetramerous flowers in which there were only 15 stamens, the missing stamen was antesepalous in 55, antepetalous in 12, and a member of the outer whorl in 6.

In 45 flowers in which more than one stamen was wanting 67 of the missing stamens were antesepalous, 25 antepetalous and 27 were from the outer whorl. The missing stamens often belonged to one or more *groups* (Figs. 23, 24): outer whorl stamens alone were rarely missing. Thus of the 27 missing outer whorl stamens the positions of 18 (in 12 flowers) were adjacent to those of antesepalous or antepetalous stamens also missing.

In 3 flowers out of the 131 recorded the symmetry of the andrœcium had been thoroughly disturbed and the stamens rearranged so that it was impossible to say that any *particular* stamens were missing.

Out of 911 flowers with 4 petals and the normal number of 16 stamens, 31 (3·4%) showed deviations from normal in the symmetry of the calyx or epicalyx or both. On the other hand, out of 207 flowers with 4 petals and some number of stamens other than 16, as many as 15 flowers (7·2%) showed deviations in these outer members of the perianth. These deviations, however, were not necessarily in the same direction as those of the andrœcium. In such cases there seems to have been a general disturbance in the development of the flower leading to deviation from normal symmetry in epicalyx and calyx on the one hand, and andrœcium on the other, but not specifically connected as in the cases now to be described.

Reduction in receptacular sectors. The typical number of stamens in many normally pentamerous species of *Potentilla*, as well as in the tetramerous and trimerous flowers of *P. erecta*, is 4*n*, where *n* is the number of petals, yet by far the commonest number of stamens in pentamerous flowers of this species is 18 (Fig. 17) and not 20. In the majority of these flowers with 18 stamens the space between the insertions of the two petals opposite the region in which the stamens are reduced in number is less than the spaces separating the other petals, so that the blades of these two petals often overlap and the corresponding antesepalous stamen and the two adjoining stamens of the outer whorl are replaced by a single stamen (Fig. 17). In a certain proportion, too, of these flowers the sepal opposite the approximated pair of petals is reduced in size. In a smaller number the two members of the epicalyx on each side are also reduced (Figs. 4, 17). Similarly, a certain proportion of flowers with 4 petals have two of them approximated and a corresponding reduction of the andrœcium to 14 stamens, while reduction of a sepal or of a sepal and the two members of the epicalyx on each side may occur here also (Fig. 30).

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* The *positions* of the stamens in the remaining flowers out of the 1176 whose stamen *numbers* were counted were not recorded.

These phenomena present us with truly intermediate stages between successive types of perfect actinomorphic symmetry, and will be further dealt with in the description below of the series of these types and the transitions between them. The obvious explanation is that in a certain sector of the receptacle, usually antesepalous, reaching from its edge or near its edge to the outer limit of the gynæceum or beyond, there has been failure of development, or of full development, leading to reduction or disappearance of the members of successive whorls situated on its central line (usually an antesepalous radius) and approximation of those on each side of that line.

The gynæceum

Table 3 shows the number of carpels in relation to the different types of symmetry.

Table 3. *Numbers of carpels in flowers with different types of symmetry*

No. of carpels ...	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Av. no. of carpels
3-merous flowers		3	2	6	3	2	—	—	—	—	—	—	—	—	4·9
4-merous flowers with less than 16 stamens	5	21	67	83	42	36	19	7	2	1	—	—	—	—	5·3
4-merous flowers with 16 stamens	2	29	283	220	192	188	193	39	13	6	4	1	2	1	5·9
4-merous flowers with more than 16 stamens	—	3	8	8	8	11	10	2	3	3	—	1	—	—	6·6
5-merous flowers	—	—	4	9	8	11	6	8	—	—	3	1	—	—	7·1

The figures in Table 3 bring out the fact of correlation of the number of carpels not only with the types of perianth symmetry but also with the number of stamens in the tetramerous flowers. This is not very well shown by the distribution of the *modes* of curves that can be constructed from the different lines of the table, but it appears very plainly from the figures for the average numbers of carpels found in the different types of flower, which form a regular series.

The 'typical' arrangements of carpels in the normal tetramerous flower have already been described—4 or 8 carpels arranged in a square—(p. 97, Figs. 13, 14).

The average number of carpels in 4-petalled flowers with 16 stamens is 5·9, and this may be taken as the average residual organ-forming capacity* of these flowers when the carpels come to be formed. If a normal frequency curve is constructed with the mode at 5·9 of the 1173 flowers in the third line of Table 3, and compared with the bimodal curve of Graph I which is made from the empirical data, it is clear that the normal curve is distorted by 'drags' towards 4 and 8 carpels in conformity with the tetramerism of the flower. Nearly 92% of these flowers have a number of carpels within the range 4–8, the mean (6) being very close to the average carpel capacity (5·9), the drag towards 4 being somewhat stronger than that towards 8. In Graph II, for flowers with less than 16 stamens, the average capacity is 5·3 and the single mode is at 5, the second highest point on the curve being at 4 carpels.

Pentamerous flowers with the common reduced andrœcium of 18 stamens often have 8 carpels in two whorls as in the tetramerous flower, 3 of the 4 belonging to the outer whorl being opposite the 3 normally spaced petals and the fourth opposite the narrow space separating the two approximated petals (Fig. 17). Here the gynæceum has attained tetramery, while the outer whorls of the flower are still intermediate. In other pentamerous flowers, with 9 or 10 carpels, the carpel at the inner end of the reduction sector is missing or is set in towards the centre of the flower (Fig. 16). Very frequently, however, variation in the number of carpels is apparently unconnected with that of the outer whorls.

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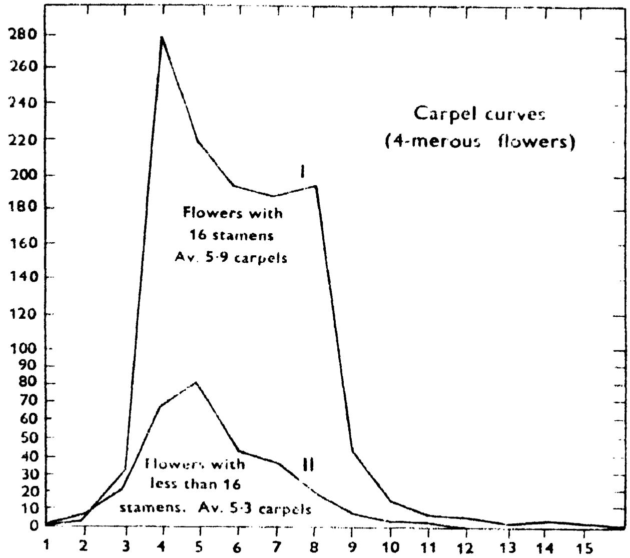
* See the remarks in the postscript at the end of this paper.



Graphs I and II. Numbers of carpels (abscissae) in flowers with 16 stamens (I) and with less than 16 stamens (II). Ordinates: numbers of flowers. See text, p. 100.

STAGES OF TRANSITION FROM HEXAMEROUS THROUGH PENTAMEROUS AND TETRAMEROUS TO TRIMEROUS FLOWERS

The hexamerous flower (Fig. 1)

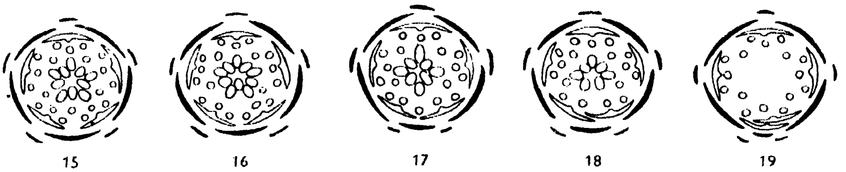
We found no case of perfect actinomorphic symmetry among 18 hexamerous flowers examined. None had as many as 24 stamens, the full complement of an actinomorphic hexamerous flower. The numbers ranged from 22 down to 14, the average being 18·5. The modal number was 18, represented in 5 flowers. The reduction in the number of stamens from the ideal 24 is brought about by the replacement of an antesepalous and the two adjacent outer whorl stamens by one stamen of the outer whorl, in a single sector or in more than one, together with absence of other stamens. It is noteworthy that in the hexamerous flowers the average number of stamens, though 0·5 greater than in the pentamerous flowers, falls much farther below the full complement of a perfect actinomorphic flower than it does in the lower types of symmetry.

The hexamerous flower is the rarest of the four types of perianth symmetry occurring in tormentil flowers. Hexamerous symmetry is a plus variation from the pentamerous type of the genus, but none of the hexamerous flowers we saw had been able to 'keep it up', so to speak, and complete the actinomorphic plan. One flower indeed (Figs. 2, 27) had a hexamerous calyx and epicalyx but failed to develop more than 5 petals and 19 stamens, including an anomalous extra stamen. Here the total 'organ-forming capacity' (or alternatively the available receptacle space, see Postscript, p. 109) had begun to fall short after the calyx had been laid down.

The pentamerous flower

Out of a set of 63 flowers with 5 sepals and 5 petals the average number of stamens was 18: only 4 had 20 stamens. In one of these perfect actinomorphic symmetry was completed by 2 whorls of 5 carpels each (Fig. 15). Another flower with 5 petals, as already mentioned, had 6 sepals and six members of the epicalyx (Figs. 2, 27). In a third flower with 20 stamens two of the petals were approximated, showing the beginning of reduction in an antesepalous sector without affecting the number of any of the floral members. Thirty-seven flowers showed further stages of reduction in a single antesepalous sector.

Of these 37 flowers 5 had 19 stamens, one antesepalous stamen being absent in each case (Fig. 16). In two of these the corresponding sepal was reduced, and in one the two petals on each side were approximated.



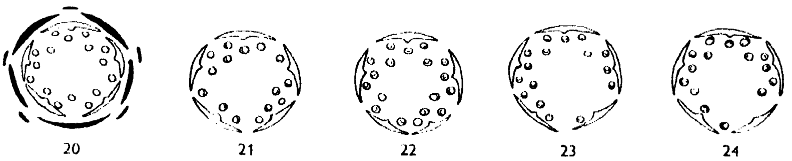
Figs. 15–19. Series showing stages of reduction from 20 to 16 stamens in an antesepalous sector (represented as anterior): perianth pentamerous throughout. Fig. 15. 'Eucyclic' pentamerous flower (20 stamens 10 carpels). Fig. 16. Antesepalous stamen wanting, corresponding antesepalous carpel displaced towards centre of flower (19 stamens, 10 carpels). Fig. 17. Two outer whorl stamens replaced by one, two petals approximated, sepal reduced two members, of the epicalyx reduced (commonest type, 18 stamens, 8 carpels). Fig. 18. Outer whorl stamen wanting, two petals overlapping (17 stamens, 7 carpels). Fig. 19. Two antepetalous stamens replaced by one, two petals extensively overlapping, sepal very much reduced (16 stamens, as in typical tetramerous flower).

Twenty-two flowers of the 37 had 18 stamens and in all of these one antesepalous stamen and the two adjacent outer whorl stamens were replaced by a single outer whorl stamen, the petals on each side being approximated (Fig. 17). In 12 of these 22 flowers one sepal and the two adjacent members of the epicalyx were reduced (Fig. 17), and in 4 the sepal alone was reduced. In 2 flowers an adjacent member of the epicalyx was absent and in one of these the sepal was also absent.

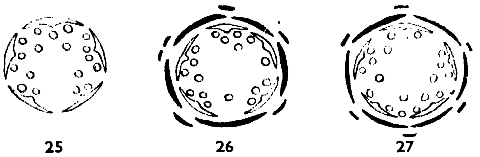
Seven flowers of the 37 had 17 stamens, the outer whorl stamen usually present on the radius of reduction being absent: in four of these two petals were approximated, their blades overlapping, and in one the corresponding sepal and the two adjacent members of the epicalyx were reduced (Fig. 18): in the other three two petals were replaced by one and the corresponding sepal was absent, so that the flower was actually tetramerous in perianth though the andrœcium was of the reduced pentamerous type.

Three flowers of the 37 had 16 stamens and in two of them two petals were very closely approximated, so that their blades overlapped extensively, and the two antepetalous stamens belonging to them were replaced by one, so that here the andrœcium was tetramerous while the corolla remained pentamerous: in one of these flowers the corresponding sepal was very much reduced (Fig. 19); while in the third flower, in addition, one adjacent member of the epicalyx was absent and the other reduced.

In the 22 remaining pentamerous flowers out of the whole set of 63 the number of stamens varied from 15 to 19, but the reduction was not confined to one radial sector as in the flowers just described. Occasionally this type of reduction was found in two distinct radial sectors (Fig. 20), sometimes it was accompanied by the absence of various other stamens (Fig. 21). In other cases more than one antesepalous, or antesepalous and antepetalous, stamens were missing (Figs. 22, 26). More rarely there were stamens missing from the outer whorl and in some cases a considerable group of stamens in one region was missing (Figs. 23–25). In other cases again, in which stamens were missing, a supplementary stamen (or more than one) was present in another part of the andrœcium (Figs. 25, 27).



Figs. 20–24. Various irregular andrœcial reductions in pentamerous flowers. Fig. 20. Type of reduction shown in Fig. 17 here affecting two antesepalous sectors (right and left)—16 stamens. Fig 21. Typical reduction to left, two other antesepalous and one antepetalous stamen also wanting—15 stamens. Fig. 22. One antesepalous and one antepetalous stamen wanting. Fig. 23. Group reduction on one side of the flower and one antesepalous stamen wanting besides (perianth not affected). Fig. 24. Group reduction on one side of the flower and an outer whorl stamen wanting (perianth not affected).



Figs. 25–27. Irregular development of andrœcium. Fig. 25. Antesepalous and two adjacent outer whorl stamens wanting: one antepetalous stamen replaced by two. Fig. 26. One antepetalous and two antesepalous stamens wanting. Fig. 27. Hexamerous calyx and epicalyx, pentamerous corolla: extra outer whorl stamen, an antesepalous and an antepetalous stamen wanting (cf. Fig. 2 which is the same flower).

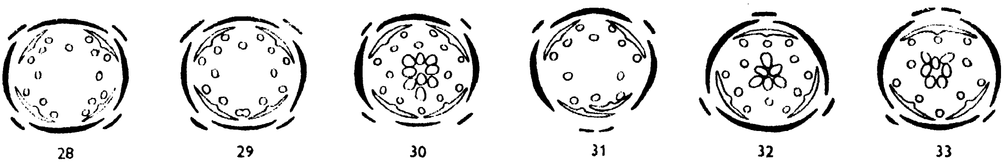
Generalizing from the details set out in the last few paragraphs one may say that the structure of the pentamerous flower is very variable but that in 38 flowers out of 63 (60 %) the variation is of a definite type, which may be considered as greater or lesser reduction in a single antesepalous radial sector, while the same type of reduction occurs in more than one sector in a proportion of the remaining flowers. In some of these flowers this is combined with the absence of one or more other stamens in other sectors, and rarely with the addition of supplementary stamens.

The tetramerous flower

The flower shown in Fig. 19 is the last connecting link we met in the series of progressive reductions of the pentamerous type leading to the tetramerous. If the two overlapping petals were replaced by one and the reduced sepal eliminated the flower would be actinomorphically tetramerous: the andrœcium is already that of the typical tetramerous flower.

While only 4 out of 63 pentamerous flowers, or 6 %, had $4n$ (20) stamens, 911 out of 1118 of the tetramerous, i.e. about 81 %, had $4n$ (16) stamens. About 17 % had a smaller number and less than 2 % a larger number than 16, so that here again we meet with far more *minus* than *plus* deviations of the andrœcium from the perfectly actinomorphic type, though *minus* deviations form a much smaller proportion of the total number of tetramerous flowers than they do of the pentamerous.

Among these *minus* deviations of the andrœcium exactly the same type of reduction is found, leading from the actinomorphic tetramerous to the actinomorphic trimerous flower, as that which leads from the pentamerous to the tetramerous. This is illustrated in Figs. 28–32. Fig. 28 shows a flower with an antesepalous stamen missing but no other change from the normal tetramerous flower. Fig. 29 shows a further stage, in which two adjacent petals are approximated and the two adjacent stamens of the outer whorl are replaced by a double staminal structure such as is represented in Figs. 11 and 12.* In Fig. 30 this is replaced by a single normal stamen and the sepal opposite it is reduced. In Fig. 31 the sepal has disappeared, the two members of the epicalyx are close together, the two petals are very closely approximated, overlapping extensively, while the number of stamens is reduced to 12, the outer whorl stamen having disappeared and the two antepetalous stamens being replaced by one, situated between the two closely approximated petals. This flower is obviously very close to the trimerous type, which the andrœcium has already attained.



Figs. 28–33. Stages of reduction from tetramerous to trimerous symmetry in an antesepalous sector. Fig. 28. Tetramerous flower with one antesepalous stamen wanting (15 stamens). Fig. 29. Tetramerous flower with two petals approximated and the two outer whorl stamens opposite their inner edges replaced by a double stamen. Fig. 30. As Fig. 29, the double stamen replaced by a single one: corresponding sepal reduced (14 stamens). Fig. 31. Two petals overlapping extensively and the two antepetalous stamens replaced by a single one: corresponding sepal wanting (12 stamens as in a trimerous flower). Fig. 32. Actinomorphic trimerous flower. Fig. 33. Reduced trimerous flower. One antesepalous stamen absent and the two adjacent outer whorl stamens replaced by one (10 stamens).

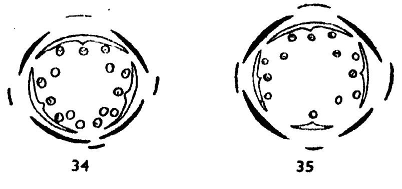


Fig. 34. Reduction in an antepetalous sector with loss of a petal and the antepetalous stamen. Fig. 35. Reduction in an antepetalous and the adjacent antesepalous sectors.

Unfortunately, the data recorded do not permit of any statement about the proportions of these successive stages of reduction occurring in the whole number of reduced tetramerous flowers, but it must be noted that some tetramerous flowers show reduction in an *antepetalous* sector, as in Fig. 34, where one petal has disappeared together with its antepetalous stamen, the sepals on each side are approximated and the corresponding member of the epicalyx is reduced. Fig. 35 shows reduction in an antepetalous and the two adjacent antesepalous sectors, the petal being reduced in size,* though its antepetalous stamen remains, and the corresponding member of the epicalyx is reduced, while the two outer whorl stamens, one on each side, are gone, together with the antesepalous stamen on one side, the sepal on the other side being reduced.

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* These double stamens are very rare in the species. They represent an intermediate stage of reduction between the presence of two adjacent stamens and their replacement by a single one, in the process of what Čelakovský (1894) called 'negative dédoublement'.

The trimerous flower

Here we find that perfect actinomorphic symmetry is common. Thus out of 21 trimerous flowers 11 had 12 stamens, and among them were flowers like that shown in Fig. 32 in which there is actinomorphic symmetry throughout, with two whorls of 3 carpels each, arranged in a triangle, just as in the normal tetramerous flower (Fig. 14) they may be arranged in a square. Of the remaining 10 flowers none had 11 stamens but three had 10, an antesepalous stamen together with the two adjacent stamens of the outer whorl being replaced by a single outer whorl stamen (Fig. 33).† Five had 13 stamens, one 14 stamens, and one 15. Thus the majority of variations in the number of stamens in trimerous flowers are *plus* deviations from the $4n$ type, and the average number of stamens is 12·3, slightly exceeding the modal number.

The flower represented in Fig. 34 is of special interest, for it is the only case we found in which the andrœcium of a flower with n petals approaches the type proper to a flower with $n+1$ petals. The calyx and epicalyx are tetramerous, and it is clear that the addition of a petal and its antepetalous stamen would make the flower tetramerous throughout. In accordance with the general tendency in the species to reduction from one actinomorphic type of symmetry to the next below, this flower is most naturally regarded as a tetramerous flower with reduction in a narrow *antepetalous sector* affecting the zone of the corolla and andrœcium alone. This is a very rare case.

'MERISTIC' AND 'CONTINUOUS' VARIATION

In his *Materials for the Study of Variation* (1894) Bateson drew attention to what he called 'meristic' variation, i.e. changes in the number of parts or members of a linear or a radially symmetrical series, of which the vertebrae of the spinal column and the members of a perianth are respective examples. In a joint paper with his sister (1891) he had previously recorded cases of the appearance of new types of zygomorphism in *Linaria*, *Veronica*, and *Streptocarpus* connected with changes in the number of parts in some of the floral whorls. The authors insisted on the rarity or absence of intermediate forms connecting the deviating types of symmetry with the normal forms of these flowers. In *Potentilla erecta* (as in many other actinomorphic flowers) there is, as we have seen, a considerable amount of 'meristic variation in a radial series', but so far from there being no intermediate forms connecting the deviating types of actinomorphic symmetry, the existence in the tormentil of a whole series of such forms is the most striking feature of the scheme of variation. Starting from an actinomorphic form with the greater number of members in the floral whorls we proceed from step to step of this series by the progressive 'cutting out', so to speak, of a wider and wider antesepalous sector of the receptacle, until the gradually approximated antepetalous sectors on each side become one, and the next type of actinomorphic symmetry is reached. In making this passage from one actinomorphic type to the next we have passed through a series of forms which are symmetrical only about the plane on each side of which the changes are taking place—flowers which are technically zygomorphic and which occur as variations from a normal actinomorphic type connected with changes in the numbers of parts in the floral whorls. This is the case mentioned by the Batesons as one which had not yet been found, but which might indicate a mode of origin of zygomorphic flowers in phylogeny. By far the commonest of these intermediate zygomorphic forms in the tormentil is the pentamerous flower with 18 stamens, 2 approximated petals and a reduced sepal (Fig. 17). Back views of this type are shown in Figs. 4 and 5.

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* Reduction of a petal in size is very rare in the species.

† This represents the most reduced type of symmetry met with, though one of the flowers had (surprisingly) as many as 7 carpels (Fig. 33).

The transitional stages, recorded in Figs. 16–19 and 28–31, represent, for the transitions in question, a series of small intervals, which may be regarded as minimal intervals in terms of the gradual curtailment of a receptacular sector. Passing down the series the members which lie on the radius about which reduction takes place undergo gradual reduction in size (sepal), or sudden disappearance (antesepalous stamen), while the members on each side of this radius, i.e. those belonging to whorls which alternate with the sepals—the two members of the epicalyx, the two petals, the two stamens of the outer whorl, and the two antepetalous stamens—undergo gradual approximation and eventual replacement, in each case, of the approximating pair of members by a single one. A 'double' organ is intermediate between a pair of members and a single member, and we met with rare examples of stamens showing varying degrees of doubleness occupying the position of pairs of stamens in reduced tetramerous flowers (Figs. 11, 12 and 29).*

With regard to the 'sudden' disappearance of an antesepalous stamen (which, together with the approximation of the two members of a pair of organs on each side of the antesepalous radius, is the first step in the progressive reduction) it might be argued that here is a gap in the series—that we ought, supposing the variation to be continuous, to find the stamen gradually reduced in size like the corresponding sepal. To this it may be replied that the progressive reduction of a sector of the developing receptacle, which we are supposing to be the proximate cause of the change in symmetry, may be continuous, but that its effect on the development of a stamen, unlike its effect on a sepal, would be discontinuous. The sepals are a whorl of leaf-like organs whose broad bases are in lateral contact, occupying together the whole circumference of the receptacle. If this circumference is abbreviated in the region of the base of a sepal the direct effect will be abbreviation of that base and thus a reduction in size of the whole sepal. This is what actually occurs. The stamens, on the other hand, arise as separate rudiments on a flat surface or slightly projecting ridge. The bases of the individual adult stamens are not in contact, and each occupies quite a small space on the surface of the receptacle. It is not, therefore, difficult to understand how a very small reduction of the staminal zone on an antesepalous radius might lead to entire failure of a stamen to develop.

REDUCTION IN THE FLOWER OF THE TORMENTIL

It is generally agreed by floral morphologists that one conspicuous tendency in the evolution of flowers is a change from spirally arranged to whorled floral members, followed by reduction in the number of whorls and in the number of members of certain whorls, often of the andrœcium and particularly of the gynæceum. In many Rosaceae, however, a pentamerous perianth is followed by more than one whorl of stamens whose numbers are decidedly variable and often by an 'indefinite' (i.e. very variable) number of spirally arranged carpels. This is true of most species of *Potentilla.* In *P. erecta,* the whole symmetry of the flower is typically reduced to tetramery, and the closely allied *P. procumbens* also has many tetramerous flowers. The andrœcium has three well-defined whorls, and the gynæceum, while sometimes retaining a pleomery of spirally arranged carpels, was found to have, out of 1173 flowers with tetramerous symmetry and 16 stamens, as many as 476 (40%) with one whorl of 4 or two whorls each of 4 carpels, while 1076 out of the 1173 flowers (nearly 92%) had 4, 5, 6, 7 or 8 carpels—representing the attainment of a typically whorled though still very variable gynæceum.

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* There were also occasional, apparent but not quite unequivocal, instances of double petals.

The number of stamens is also very variable, but the variation, as we have seen, follows for the most part very definite lines, the great majority of deviations from the number which would accord with the general symmetry being *minus,* except in trimerous flowers, and often giving an *even* number of stamens with a preponderance of $4n-2$, where n is the number of petals. This is true of a large proportion of pentamerous flowers and of a substantial number of those tetramerous flowers which have less than 16 stamens. As we have seen, this condition is usually brought about by the replacement of an antesepalous stamen and the two adjacent stamens of the outer whorl by a single stamen of the outer whorl. This arrangement of stamens thus appears to be what Galton called a 'position of organic stability', subsidiary, as such a position, to the arrangement in the normal tetramerous flower ($4n$) but exceeding in frequency the $4n$ number in the pentamerous flower. In the trimerous flower the deviations of the andrœcium are on the *plus* side. Thus the number and arrangement of stamens in the andrœcium is, as it were, 'pulled' towards that characteristic of the normal tetramerous flower in the pentamerous types on the one side and the trimerous types on the other. There are, however, other deviations from the normal type which do not fit into this scheme—reductions in antepetalous sectors and 'group' reductions.

The reduction of organs situated on an antesepalous (more rarely on an antepetalous) sector of the receptacle often affects, as we have seen, the whole length of the sector from epicalyx to gynæceum, but sometimes single or two successive zones alone. The particular sensitiveness of the andrœcium may depend on variations in the development of the staminal zone of the receptacle after the perianth, or at least the calyx, has been laid down (cf. Goebel, 1883). The zone that gives rise to the petals is often involved with the staminal zone,* as is shown by the approximation of two petals accompanying the replacement of three stamens by one in an antesepalous sector, which may, or may not, be accompanied by reduction of the corresponding sepal. The gynæceum is usually also involved, as is shown by the fairly close correlation of the number of carpels with the number of stamens, but sometimes it is not, since it shows variations apparently unconnected with those of the andrœcium.

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* The close relationship in development of petals and stamens is well known in many flowers of varying affinity.

SUMMARY

1. The flower of *Potentilla erecta* is typically tetramerous and cyclic throughout, the gynæceum, in 40% of the whole number of tetramerous flowers, consisting of one or two whorls of 4 carpels each or some slight deviation from this arrangement (51%) with 5, 6 or 7 carpels. There is an outer whorl of 8 stamens, a middle one of 4 antepetalous, and an inner of 4 antesepalous stamens. The typical formula is thus Ak 4, K 4, C 4, A 8+4+4, G 4, or 4+4.

2. The symmetry of the perianth, however, ranges from hexamerous to trimerous and in collections of many thousands of flowers the total deviation from tetramery of the perianth amounts to between 3 and 4%. Hexamerous and trimerous flowers are both rare, but pentamerous number about 3% of any large collection except at the beginning of the flowering season when the proportion may be considerably higher.

3. Hexamerous flowers with completely actinomorphic arrangement have not been met with, the number of stamens ranging from 22 to 14, whereas in a completely actinomorphic hexamerous flower there should be 24.

Pentamerous flowers are sometimes completely actinomorphic (A = 20) but in most cases show a reduction, occasionally to 19, but most commonly to 18, by the absence of an antesepalous stamen and the replacement of the two adjacent stamens of the outer whorl by a single one. Other pentamerous flowers have a still further reduced andrœcium. In reduced pentamerous flowers the two petals opposite the locus of reduction in the andrœcium are often approximated, and the corresponding sepal is sometimes reduced in size, while a cyclic arrangement of the carpels may be disturbed on the same radial sector. A complete series of transitional stages between the pentamerous and the tetramerous types has been found.

The overwhelming majority of tetramerous flowers are completely actinomorphic, but a few (1·7%) have a larger, and 16·8% a smaller number of stamens than 16. The type of reduction in the andrœcium is mostly the same as in pentamerous flowers, i.e. it occurs in antesepalous sectors, usually simply by the absence of an antesepalous stamen, sometimes by a further reduction in the same sector; but occasionally the reduction is in an antepetalous sector. Less than 10% show a number of carpels outside the range 4 to 8. There is a complete series of transitional forms between actinomorphically tetramerous and actinomorphically trimerous flowers.

About half the trimerous flowers examined were actinomorphic throughout, and of the other half most showed *plus* variations in the andrœcium (not *minus* as in the other types of symmetry). Thus there are 3-petalled flowers whose andrœcium shows transitions towards the tetramerous type as well as 4-petalled flowers showing transitions towards the trimerous.

4. If we make the obvious assumption that the flower of the tormentil is derived from the pentamerous type characteristic of the genus *Potentilla* it is evident that tetramerous symmetry is firmly established in it and that very few completely actinomorphic pentamerous flowers now remain within the range of variation of the species, while there is a considerable number of pentamerous flowers which show transitions towards tetramery. Within the range of variation the process of reduction has gone beyond the actinomorphic tetramerous type and is represented by flowers with fewer than 16 stamens showing transitions towards trimery, and by actinomorphic trimerous flowers, while there are

a few trimerous flowers showing still further reduction of the andrœcium down to 10 stamens. The symmetry of the perianth, as established at the outset of development, is of course the main determinant of the number of stamens and carpels, but deviations from the 'typical' number corresponding with strictly actinomorphic arrangement are numerous and are mainly in a *minus* direction in all types except the trimerous, emphasizing the general tendency to reduction which passes beyond the tetramerous condition, and even occasionally beyond actinomorphic trimery within the range of variation of the species.

5. Since reduction of all the whorls is, in the majority of cases, in an antesepalous sector, and there is a complete series of these from the actinomorphic pentamerous to the actinomorphic trimerous flower, and beyond, it is difficult to avoid the conclusion that its mechanism depends on a progressive curtailment of growth of the developing receptacle, usually in one of these sectors.

POSTSCRIPT

Mr I. H. Burkill has been kind enough to read the typescript of this paper. He envisages the basic cause of reduction in the symmetry of a flower as restriction of the vigour or capacity to produce the full number of floral organs characteristic of the unreduced flower. Thus the actinomorphic pentamerous flower of the tormentil with 10 carpels (Fig. 17) (K 5, C 5 A 10+5+5, G 5+5) would have a total capacity of 40 (omitting the epicalyx), exceeding that of the tetramerous flower by 8. If the stamens are reduced to 18 and the carpels to 8 (Fig. 17) the capacity is only 36. The tetramerous flower with 8 carpels (Fig. 14) has a capacity of 32, with 4 carpels (Fig. 13) only 28, while the trimerous type with 6 carpels (Fig. 32) has a capacity of 24. Defective capacity, falling below that of a eucyclic flower, often shows itself in the failure to produce the full number of the later-formed organs required by the eucyclic scheme. This may become fixed in the species, as in the frequent typical oligomery of the gynæceum in otherwise eucyclic flowers belonging to plants of very various affinity; and this fixed reduction may extend to the andrœcium. But a flower must produce all categories of floral organs if it is to function normally: if its total organ-forming capacity is reduced it cannot use it all up in producing the outer whorls alone and have none left for the carpels. So that when the total capacity falls below some critical limit the number of members in each whorl is reduced and a lower type of symmetry established. This reduction is initiated at the outset of development with the formation of the sepals.

There is, however, another factor to be considered. The developing flower must not only possess sufficient vigour to produce a full complement of organs, there must also be enough space on the surface of the receptacle to give them room to develop, and this will also be affected by the total developmental capacity of a particular flower bud. The object of the present paper is to show that in the tormentil flower restriction of the available receptacular area, and therefore of its circumference (or the reduction of total organ capacity, or both) is *localized*, typically though not invariably, in an antesepalous sector of the flower. This is demonstrated by the complete series of intermediate conditions, representing progressive reductions in such a sector, between the pentamerous, tetramerous and trimerous actinomorphic types which occur within the observed range of variation.

REFERENCES

I have not been able to find, or to hear of, any work published in recent years bearing directly on the topic of this paper. The copious modern work on floral development is concerned with other aspects of the subject.

BABINGTON, C. C. (1833). On the distinctions between the Linnean genera *Potentilla* and *Tormentilla*. *Loudon's Magazine of Natural History*. Vol. VI, p. 248. London.

BATESON, W. (1894). *Materials for the study of Variation.* London.

BATESON, W. & BATESON, ANNA (1891). On variations in the floral symmetry of certain plants having irregular corollas. *J. Linn. Soc. (Bot.)*.

ČELAKOVSKY, L. J. (1894). Das Reductionsgesetz der Blüthen: das Dédoublement und die Obdiplostemonie. *S.B. böhm. Ges. Wiss.* pp. 1–142.

EICHLER, A. W. (1875–8). *Blüthendiagramme.* Leipzig.

GOEBEL, K. (1883). Vergleichende Entwicklungsgeschichte der Pflanzenorgane. *Schenck's Handbuch der Botanik*, Breslau.

PAYER, J. B. (1857). *Traité d'organogénie comparée de la fleur.* Paris.

STUDIES IN BRITISH PRIMULAS

II. ECOLOGY AND TAXONOMY OF PRIMROSE AND OXLIP (*PRIMULA VULGARIS* HUDS. AND *P. ELATIOR* SCHREB.)

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(With 3 figures in the text)

In the first paper in this series (Valentine, 1947) experiments were described on hybridization between the Oxlip (*Primula elatior* Schreb.) and the Primrose (*P. vulgaris* Huds.). In the present paper, an account is given of a hybrid population; the status of each species is assessed, and a comparison is made with other species pairs which have been investigated by the methods of experimental taxonomy.

I. Introduction

(*a*) *Geographical distribution of Primrose and Oxlip*

In this account of the Oxlip we shall be dealing with the var. *genuina* Pax; there are a number of other forms, described by Pax & Knuth (1905), which inhabit southern and eastern Europe, and which are probably of subspecific status. *P. elatior* var. *genuina* is a plant of Central Europe, and is frequent in Germany and Switzerland; it extends from England to southern Russia, and from southern Sweden to the north-west Carpathians. The Primrose, on the other hand, is a species of western and southern Europe, and is absent from central Germany; it occurs from southern Sweden to central Spain in the west, and from Italy to Syria in the south; and it is found in the Alps and on the southern slopes of the Carpathians. Hegi (1931) regards the Primrose as a thermophilous species which requires a relatively mild winter and spring; the Oxlip can withstand the low winter temperatures of continental Europe. This is supported by the fact that the Primrose in Europe is generally a lowland species, and the Oxlip a highland species.

In Britain, the Primrose is recorded from every vice-county of the British Isles, and is common in many places. The Oxlip is only found in eight vice-counties, all of which are in East Anglia; its distribution was first thoroughly worked out by Christy, and this work has formed the basis of our investigations. Christy showed that the Oxlip was confined to two regions, a smaller to the west of Cambridge and a larger, situated mainly in Essex and Suffolk. Full details, with maps, are given in Christy's papers (1897, 1922*a*, 1924). Within the Oxlip regions, Primrose is absent; only round the edges of the regions do Oxlips and Primroses meet, and here hybrid populations are found; outside the Oxlip regions, only Primroses occur. It may be mentioned in passing that the Cowslip (*P. veris* L.) is found throughout the whole area, in proximity to both Oxlips and Primroses; hybridization involving the Cowslip will not be discussed here, but it can be said that Oxlip-Cowslip hybrids are local and rare.

(*b*) *Habitat of Primrose and Oxlip*

Hegi (1931) states that in Europe the Primrose is found in fairly rich soils, usually in shady places or woodland that is not dense, but occasionally in meadows. The Oxlip is said to occur in both meadows and woodlands, but its preference for moist habitats is clearly indicated; the terms 'Auenwald', denoting a damp woodland liable to flooding, and 'Flussauen', denoting marshy meadows, are both used in describing its habitats. In Normandy, according to Corbière (1894), the Primrose is found in woods and hedges, the Oxlip in damp woods or shady meadows. Rouy (1913), for the whole of France, gives woods and meadows as habitats for both species, but adds that the Oxlip is also found in highland woods. The general conclusion from these and other floras is that both species occur in woodlands and meadows, and that the Oxlip has a special preference for moist habitats.

In Britain, Christy (1897) was able to show that the Oxlip was confined entirely to one geological formation, viz. the glacial deposit known as the chalky boulder clay; and subsequent observations have fully confirmed this. Christy further showed that the Oxlip was a woodland plant; only rarely, in about a dozen stations, many of which are detached from the main area of distribution, does it occur outside woodland; such habitats are in marshy meadows, and always on the chalky boulder clay. The Oxlip is not confined on the Continent to calcareous soils; indeed Drude (1912) remarks that it is common on siliceous soils. This difference may be taken as an example of the phenomenon, noted by Salisbury (1920), that at the climatic limit of the range of a species, soil preferences tend to become more clearly defined. As regards the preference of the Oxlip for moist habitats, British and Continental observations agree; for the boulder clay on which the Oxlip occurs in Britain is a wet soil liable to winter flooding, although it may become fairly dry during summer.

The Primrose in Britain is found on a wide range of soil types, and though it may be found on calcareous soils, it is by no means confined to them. Its distribution, however, does appear to be bound up with soil moisture and atmospheric humidity; thus in the central region of East Anglia, Primrose is mainly if not entirely found in woodland and not in hedgerows; in the west of England, where both precipitation and humidity are higher than in East Anglia, it is common in more exposed habitats. Good (1944) has studied the distribution of the Primrose in Dorset; he finds that on the heavier soils, especially the clays, Primrose occurs irrespective of rainfall, but that on lighter soils, it is only found where a relatively high precipitation gives them an adequate water-content. The effect of soil is least marked in woodland, but well marked in the more exposed hedgerow habitats.

(*c*) *The western Oxlip region in Britain*

Some additional data on the distribution of the Oxlip have been obtained since Christy (1922*a*) published his map. In this investigation, we have concentrated our attention on the smaller western Oxlip region, and a revised distribution map for this region is given in Fig. 1. The main boundaries coincide closely with those given by Christy, but there has been an extension southwards to include the Oxlips in Buff Wood, and in Potton Wood (Little, 1935). All occurrences of Primrose and Oxlip in this region are, so far as is known, in woodland, with one exception; H. & D. Meyer (personal communication) have discovered Oxlips in hedgerows and short scrub in a small area adjoining Eltisley Wood (these authors are preparing a more detailed distribution map of the region).

Fig. 1 shows, in the northern part of the region, six Oxlip woods, two of which contain Primroses as well (in very small numbers in Knapwell Wood); a Primrose wood is found at Papworth, and there are more Primrose woods on boulder clay and on Oxford clay farther to the north-west. In the southern part of the region there are eight Oxlip woods, five of which contain Primroses as well, and one Primrose wood, on boulder clay; beyond the woods, along the southern borders of the region, the boundary of the boulder clay is quickly reached. It can be seen that the woods lie on a plateau; the rock which underlies the thick coat of boulder clay consists, in different places, of chalk, gault,

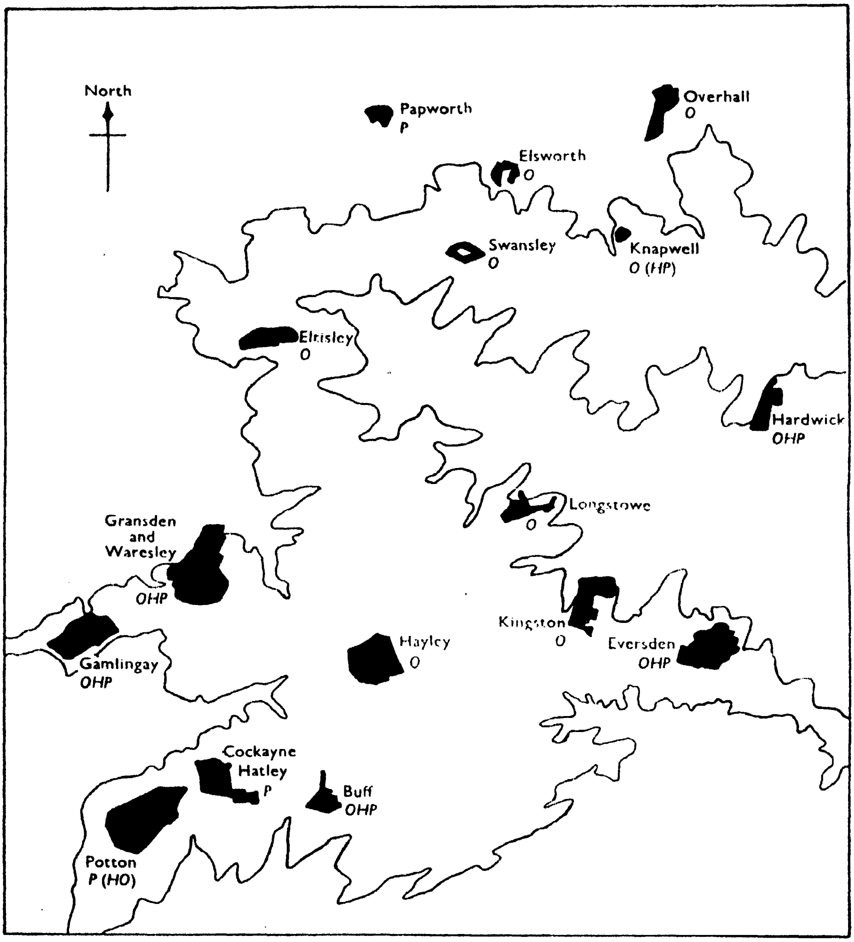

Fig. 1. Map of the western Oxlip region (½ in. to the mile), showing the position and names of the main semi-natural woods, and their relation to the 200 ft. contour; plantations are omitted. Cambridge lies about 6 miles east of Hardwick Wood. *O*=only Oxlips present; *P*=only Primroses present; *OHP*=Oxlips, Hybrids and Primroses present; *O*(*HP*) and *P*(*HO*) denote marked predominance of Oxlips and Primroses respectively.

Kimmeridge clay, Corallian clay and greensand. The composition of the drift does not appear to be affected by the nature of the rock except at the south-western border. Here, in Gamlingay and in Gransden and Waresley woods, in which the rock is gault in some parts of the woods and greensand in others, the drift overlying the greensand is modified into a non-calcareous loam; on this soil, Primroses occur but Oxlips are absent (Adamson, 1912).

The woods marked in Fig. 1 are ash-oak woods, and are probably the remnants of the primitive woodland cover of the district. Many small plantations are also found; these are not marked on the map. They consist mainly of elms, which are sometimes coppiced; the ground flora is poor in species, and often includes weeds of disturbed ground, such as *Anthriscus silvestris* and *Urtica dioica*, in abundance; most of them have been explored and Primrose and Oxlip are uniformly absent.

It is convenient at this point to summarize and review information so far presented. Central East Anglia is an area of low precipitation with a climate approximating to a continental type (Watt, 1938); it is thus a region in which it might be expected that a species such as the Oxlip, with its 'continental' type of distribution, would occur. The almost complete restriction of the Oxlip to woodland in this area appears to be due to its need for a soil of high water-content; this is provided in the boulder clay woods, which may be regarded as undrained islands lying in a region of drained, agricultural land. The Primrose, on the other hand, has an 'Atlantic-Mediterranean' distribution, and is apparently favoured by mild winter conditions; it is thus unfavourably situated in the quasi-continental area of central East Anglia, and is able to survive only within woodland, where it is sheltered to some extent from winter frosts. Thus, the restriction of both species to woodland in the area concerned would be due, with Oxlip, to edaphic factors, and with Primrose, to climatic factors.

An important corollary of these facts is, that migration of the species between woods is not taking place at the present time. The only likely means of migration is by seed; Oxlip seeds are dispersed by a 'censer' mechanism, Primrose seeds by ants; it is unlikely that migration by such means could take place without traces of the passage of the plants being left in the areas between the woods. It follows that, under present conditions, woods containing Oxlips only are not likely to be invaded by Primroses, and vice versa.* This point bears on the hypothesis of Christy (1922*a*), that the 'aggressive' Primrose is tending to oust the diminishing Oxlip. The question as to whether or not this is happening in woods which now contain both species will be discussed below; but it is fairly clear that, as between wood and wood, it is not happening at present. It might be said that the Primrose and Oxlip populations of the individual woods were 'micro-geographically' isolated from one another. Populations of other woodland species, e.g. *Viola riviniana* (Valentine, 1941), are similarly isolated.

II. ECOLOGY OF OXLIP AND PRIMROSE IN BOULDER CLAY WOODLAND

The type of wood in which Oxlips are found has been described by Adamson (1912) and Godwin (1938). The wood generally consists of standards of oak (*Quercus robur*) with hazel coppice; standard ash and elm (*Ulmus minor* Mill.) often occur, and ash and maple are frequent among the coppiced shrubs. There is a wide variety of other woody plants, and a rich ground flora which contains many calcicoles.

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* Planting of either species by man would alter the case, and this possibility cannot be ruled out. We have evidence that Primrose seed has been sown in at least one wood in the Oxlip region.

The boulder clay is stiff; it contains flints and an abundance of chalk. The layer of humus at the surface is shallow, and it is difficult to make out any well-marked profile. The pH of the soil has not been systematically investigated, but a number of spot determinations, on samples collected from the top 5 cm. of soil at scattered localities throughout one wood, have been made; of the sixteen samples examined, ten had a pH between 6·6 and 6·9; the range for all the samples was 4·7–8·0. There is a well-marked seasonal fluctuation in the water-content of the soil, data on which are given below (p. 119); in late winter and early spring, the soil surface appears to become saturated locally, with free water in the surface layers.

When inquiring into differences between Primrose and Oxlip habitats, it is of interest to compare communities containing only the one species with communities containing only the other. In Tables 1 and 2 are given data for plant societies, recorded in a number

Table 1. *Lists of selected species (mainly the more constant species) from Oxlip woods. Each list refers to a single characteristic society in the wood*

	E.(*b*)	E.(*a*)	S.	L.	K.	O.(*b*)	O.(*a*)	Hl.	H.(*a*)	H.(*b*)	O.(*c*)
Primula elatior	o., l.a.	a.	a.	a.	f.–a.	o.	a.	d.	a.	f.	a.
Mercurialis perennis	d.	a.	a.	a.	f.	—	—	—	—	—	—
Scilla non-scripta	o.	l.f.	l.f.	o.	f.	v.a.	a.	—	l.a.	—	—
Arum maculatum	o.	f.	l.f.	f.	f.	f.	f.	—	f.	—	f.
Ranunculus ficaria	a.	o.	—	—	r.–f.	v.a.	v.a.	—	f.	a.	a.
Anemone nemorosa	—	—	o.	—	l.f.	—	—	o.	f.	+	—
Glechoma hederacea	—	o.	—	—	o.	—	—	o.	f.	—	+
Ajuga reptans	—	o.	f.	o.	—	—	—	o.	—	—	—
Filipendula ulmaria	—	—	—	—	—	—	—	f.	l.f.	f.	+

E. Elsworth Wood, Cambs., 12. iv. 38. (*a*) Dense hazel coppice, 15 ft., slight slope; (*b*) elm and hazel, 30–60 ft.

S. Spriggs Wood, Essex, 7. iv. 37. Elm and hazel coppice, 15–30 ft.

L. Langley Wood, Essex, 18. iii. 37. Hazel and ash coppice, 12 ft.

K. Knapwell Wood, Cambs, 22. iv. 37. Dense mixed scrub, mainly blackthorn.

O. Overhall Grove, Cambs, 22. iv. 37. (*a*) Elm, 30–50 ft., on slope, (*b*) as (*a*); (*c*) elm coppice, 12–15 ft.

Hl. Hales Wood, Essex, 28. v. 37. Hazel and ash coppice, 12–20 ft.

H. Hayley Wood, Cambs, 9. iv. 37. (*a*) Hazel coppice, 15–20 ft.; (*b*) in gap in coppice. + = present, but frequency not recorded.

Table 2. *Lists of selected species (mainly the more constant species) from Primrose woods. Each list refers to a single characteristic society in the wood*

	L.P.	B.	P.	C.
Primula vulgaris	f.	f.	a.	f.
Mercurialis perennis	f.	f.	—	—
Scilla non-scripta	a.	f.	f., l.a.	o.
Arum maculatum	o.	l.f.	f.–a.	—
Ranunculus ficaria	—	l.f.	a.	f.
Anemone nemorosa	—	—	f.	a.
Glechoma hederacea	—	—	o.	—
Ajuga reptans	—	l.f.	f.	—
Filipendula ulmaria	—	—	o.	—

L.P. Little Paxton Wood, Hunts, 20. iii. 37. Ash-maple-hazel coppice, 10–20 ft.

B. Brampton Wood, Hunts, 20. iii. 37. Standard oak, hawthorn scrub.

P. Papworth Wood, Cambs, 12. iv. 38. Elm coppice, 15 ft.

C. Cockayne Hatley Wood, Cambs, 18. iv. 37. Ash-maple-hazel coppice, 20 ft.

of boulder clay woods; data from Oxlip woods in Essex and from Primrose woods in Huntingdonshire are included. Only the more constant species and some of special interest are recorded in the tables. Each list represents the composition of a fairly uniform society, typical of a considerable region of the wood, selected for description during visits to the woods in spring. In nearly every case the community described was under tall coppice, i.e. in the shade phase of the coppice rotation.

In his account of Gamlingay Wood, Adamson (1912) distinguishes two main societies in the ground flora, one dominated by *Mercurialis perennis*, the other by *Filipendula ulmaria*. The Oxlip communities (Table 1) can be arranged so as to fall roughly into these two types; there is a group of five in which *Mercurialis* is frequent, *Ranunculus ficaria* not as a rule frequent, and *Filipendula ulmaria* absent, and a group of six in which *Mercurialis* is absent, *Ranunculus ficaria* frequent and *Filipendula ulmaria* usually present in some quantity. The four Primrose societies (Table 2) can be similarly divided. The data are rather scanty, but so far as they go, they indicate a general resemblance in composition between societies containing Primrose in Primrose woods and those containing Oxlip in Oxlip woods.

In view of the conclusion just stated, it might be expected that, in woods where both Oxlip and Primrose occur, the two species would be found together in a mixed population. It is, however, found that the species remain distinct from one another, meeting along a fairly well-defined boundary line (the question of hybridization and its effect on the purity of the species will be discussed below). Distinct populations of this kind are certainly found, for example, in Buff Wood, Potton Wood, Hardwick Wood and Gamlingay Wood (Fig. 1) and in Rockell's Wood, Essex (Christy, 1924); and this suggests that there are factors of the environment which differentiate the habitats of the species within the woods.

In order to obtain precise data, investigations were made in Buff Wood, Cambs, the area of which is 40 acres. Here, Oxlips are found in a fairly well-defined area, 100–150 yd. across, in the northern part of the wood, and they are surrounded on all sides by Primroses. Two transect lines were laid down, running approximately at right angles to one another; each of these started in the 'Primrose area', traversed the 'Oxlip area' and continued into the 'Primrose area' at the other side. Each transect was 2 ft. wide, and was divided into sections 4 ft. long; the plants in each successive section were listed, and a note made of their frequencies; percentage cover on the ground and the nature of the shrubs and trees present were also noted. The lengths of the transects were 157 and 195 yd. It is thus possible to use the data from the transects to compare the vegetation in Oxlip and Primrose areas; some of the data are plotted in Figs. 2 and 3, and some have been tabulated (Table 3). Occurrences only are used in Table 3; an estimate which takes frequency into account can be made, but it does not alter the general picture. In all, fifty-four species of flowering plant were recorded in the two transects, including the woody plants. In addition to those herbs mentioned in Table 3 and in the text, the most frequent (occurring in more than 20% of the plots) were *Rumex sanguineus* L., *Potentilla sterilis*, *Fragaria vesca* and *Viola riviniana*. Hazel formed part of the canopy in 70% of the plots, and ash in 27%. Amongst the species which are mentioned by Adamson (1912), as forming an important part of field layer communities, *Mercurialis perennis* had a total frequency of 1½%, and *Deschampsia caespitosa*, 2%. *Ranunculus ficaria* and *Anemone nemorosa* both occurred in 99% of the plots, and appeared to be uniformly frequent throughout the area.

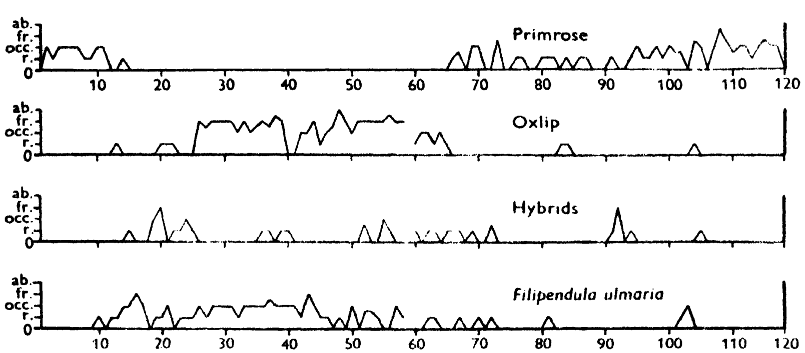

Fig. 2. Transect no. 1, through Oxlip area in Buff Wood, Cambs, April–May, 1939. The ordinate indicates frequency in the 4 × 2 ft. plots, the abscissa, the serial number of the plot. There were 118 plots, and the transect was about 157 yd. long. There is a gap in the transect at plot 59 where it crossed a narrow path. The transect ran roughly at right angles to transect no. 2 (Fig. 3). 0 = absent, *r.* = rare, *occ.* = occasional, *fr.* = frequent, *ab.* = abundant.

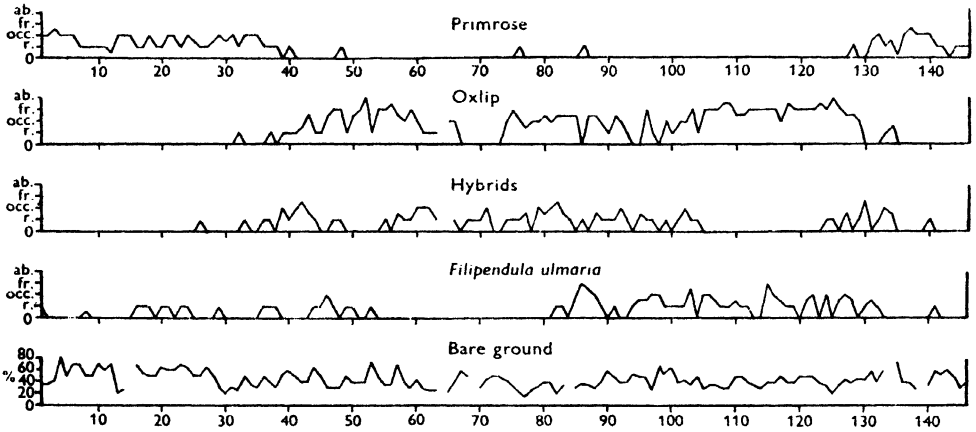

Fig. 3. Transect no. 2 through Oxlip area in Buff Wood, Cambs, April–May 1939. The ordinate indicates frequency in the 4 × 2 ft. plots, the abscissa, the serial number of the plot. There were 146 plots, and the transect was about 195 yd. long. There is a gap in the transect at plot 64 where it crossed a narrow path. The transect ran roughly at right angles to transect no. 1 (Fig. 2). The bottom curve shows the percentage of ground in each plot which was estimated to be bare or moss-covered, i.e. not covered by higher plants. 0 = absent, *r.* = rare, *occ.* = occasional, *fr.* = frequent, *ab.* = abundant.

Table 3. *Occurrences of six selected species in Oxlip and Primrose areas, Buff Wood, April–May* 1939

Results for the two transects are combined.

	Arum maculatum	*Glechoma hederacea*	*Viola reichenbachiana*	*Ajuga reptans*	*Scilla non-scripta*	*Filipendula ulmaria*
Primrose area. Total number of plots 4 × 2 ft., along the transect, which were examined = *127*						
No. of plots in which the species occurred	77	55	51	59	35	31
No. of plots in which the species occurred, as % of total no. of plots	*60·5*	*43·5*	*40*	*46·5*	*27·5*	*24·5*
Oxlip area. Total number of plots 4 × 2 ft. examined = *136*						
No. of plots in which the species occurred	83	72	62	30	36	79
No. of plots in which the species occurred, as % of total no. of plots	*61*	*53*	*45·5*	*22*	*26·5*	*58*

The agreement between the figures for *Arum maculatum*, *Glechoma hederacea*, *Viola reichenbachiana* and *Scilla non-scripta* is good; they are clearly equally distributed in both Primrose and Oxlip areas. The much lower frequency of *Ajuga reptans* in the Oxlip area is striking; the frequencies for *Ajuga* in the Oxlip area in the two transects taken separately are 42 and 12%, which suggests that the distribution of the plant, which spreads considerably by vegetative means, is patchy. The corresponding figures for *Filipendula ulmaria*, the vegetative spread of which is much more limited, are 77 and 48·5%; the respective frequencies in the Primrose area are 19·5 and 31%. There is thus evidence that *F. ulmaria* is much more frequent in the Oxlip area than in the Primrose area, and that this constitutes one of the main differences between the two areas in respect of the more frequent flowering plants. The data for *F. ulmaria* are plotted in Figs. 2 and 3.

The transects which have been described covered only a part of the wood, which contains a fairly homogeneous community under hazel coppice, and in which *Mercurialis* is rare; the slope is slight (about 1 in 50). At the south-western end of the wood, where the ground slopes more steeply (about 1 in 30), the community changes, and is dominated by *Mercurialis*; Oxlips are still absent, and Primroses persist. The composition of this community, which has been called the 'Mercury area', is indicated in Table 4.

Table 4. *Metre quadrats in Oxlip and Mercury areas, Buff Wood, April* 1940. *Only the more frequent species included. The figures refer to number of shoots*

	Mercury area	Oxlip area
Primula elatior	Nil	48
Primula vulgaris	5	Nil
Mercurialis perennis	101	Nil
Ranunculus ficaria	29	196
Anemone nemorosa	17	48
Scilla non-scripta	17	11
Arum maculatum	53	3
Filipendula ulmaria	Nil	19
A species of grass	Nil	171

The data of Table 4 show well the predominance of *Mercurialis* and the absence of *Filipendula ulmaria* from the Mercury area. It will be noticed that a species of grass is frequent in the quadrat from the Oxlip area, and absent from that in the Mercury area (the grass consisted of small vegetative shoots and it has not yet been identified, though it is probably a species of *Poa* or *Agrostis*). Analysis of the transect data showed also that a grass was markedly more frequent in the Oxlip area than the Primrose area.

We may summarize our data on these plant societies as follows:

(1) Community of Oxlip area—Oxlip abundant, *Filipendula ulmaria* frequent, *Mercurialis* rare.

(2) Community of Primrose area—Primrose abundant, *Filipendula ulmaria* occasional, *Mercurialis* rare.

(3) Community of Mercury area—Primrose frequent, *Filipendula ulmaria* absent, *Mercurialis* abundant.

It is our opinion that light intensity is not a major factor in differentiating the three communities described, but measurements of this factor have not been made. Data from the transects show that the shrub layer consisted mainly of coppiced hazel, averaging 15–20 ft. in height, i.e. the communities were in a late phase of the coppice rotation and thus, in summer, in deep shade. The rarity of *Deschampsia caespitosa*, a species considered by Adamson to be intolerant of deep shade, is significant in this respect. Standards were also rare; oak was the most frequent, forming an important part of the canopy in 9% of the plots. Figures for percentage of ground not covered by higher plants are plotted for the longer transect in Fig. 3; they show a slightly higher percentage of uncovered ground in the Primrose area than in the Oxlip area. This fact is probably correlated with the fact that Oxlips were more abundant on the ground than Primroses; how far this effect is bound up with light intensity is not known.

There is, however, some evidence that the three communities are distinguished by a difference in soil water-content. First, it is well known that *Filipendula ulmaria* is a plant which thrives in moist soils and is usually absent from dry habitats; it thus seems reasonable to regard *F. ulmaria* as a sort of biological indicator of soil moisture, and to interpret its relative abundance in the three communities as an indication of the soil conditions on which they occur. This view is supported by the data of Adamson (1912) for Gamlingay Wood. Our communities (1) and (2) correspond to some extent with Adamson's *F. ulmaria* society, which occurs in deep shade and on soil with a mean summer water-content of 36–43%; our community (3) corresponds with Adamson's Mercury society (damp type), which occurs under varying conditions of light intensity and on soil with a mean summer water-content of 25–26%. A preliminary attempt was made (in co-operation with Dr A. S. Watt) to find out if similar figures could be obtained in Buff Wood, by comparing soil water-contents in the Oxlip area and the Mercury area. The determinations were made in 1940–1, and the data, like those of Adamson, are expressed in terms of water-content as a percentage of the weight of the soil, dried at 100° C. Typical localities in the two areas were selected, and a series of samples from the top 5 cm. of soil was taken during the year. The results showed that there was a marked seasonal cycle, with a maximum of about 70% in early spring and a minimum of about 30% in September; but there appeared to be no significant difference between the figures for the two areas. The values were, on the whole, higher than those given by Adamson, but it is difficult to make an exact comparison, as Adamson states only that he collected his soil samples from those layers in which the roots of the principal species spread; he does not say specifically what these species were, nor the exact depth at which samples were taken. A further series of measurements, also during 1940–1, was made in Buff Wood, in which profiles in Oxlip and Mercury areas were sampled to a depth of 21 in.; these showed, as a rule, that the water-content of the soil decreased steadily with depth; but again, though there was an indication that the values for the Oxlip area were slightly higher than those for the Mercury area, we do not consider that the differences are significant, and further investigations are needed. We conclude, both from our own data on floristic composition and from Adamson's data on Gamlingay Wood, that there is evidence that soil water relations are amongst the factors which differentiate the communities of the Oxlip area and the Mercury area, but that data to prove this conclusively are as yet lacking.

As regards the factors which differentiate Primrose from Oxlip habitats, we have only the data of the transects and the use of *F. ulmaria* as a biological indicator to guide us. Adamson (1910) considered that Oxlip only flourishes on soil which has a water-content which is high in spring, and which never falls much below 30% at any time of the year, the lower limit probably about 25%; Primrose can do well on much drier soils. The fact that Adamson (1912) records both Primrose and Oxlip from both the *F. ulmaria* societies and the Mercury society (damp type) in Gamlingay Wood reinforces this conclusion, but does not help in the analysis of the factors affecting Primrose-Oxlip distribution in Buff Wood. Our tentative conclusion must be that soil water relations are again important; but observations continued over several seasons are needed to decide the matter.

The facts that both Oxlip and Primrose populations are fairly pure, and that the boundary between them is well marked (Figs. 2 and 3) point to the likelihood of intense competition between the species; the situation is perhaps one to which the hypothesis of Gause (1934) is applicable, namely, that closely allied species with similar ecology cannot survive together in the same place, because competition between them leads to the elimination of one or the other. It is not possible at present to judge with certainty the stability of the situation, but it may be that quite a small change in environmental conditions, for example, increased precipitation or improved drainage, might shift the boundary between Primrose and Oxlip in one direction or the other. Where there is no inter-specific competition, as in woods which contain only Oxlip or only Primrose, it would be expected that the ecological range of both species would be wider than it is, for example, in Buff Wood. The data presented in Tables 1 and 2, and discussed on p. 116, support this conclusion, in that they show that Oxlip may occur in communities in which Mercury is abundant, and in which it might well be replaced by Primrose, were Primroses present. The introduction of one of the species into a wood in which only the other species occurred would thus be an experiment of great interest. Such an experiment has been performed; Christy (1922*b*), in 1898, planted nine separate patches of Primroses in a pure Oxlip wood, Peverell's Wood in Essex; in 1915, it was found that more than one of the patches of Primroses still survived, and some interspecific hybrids had also been formed. It does not appear from Christy's account that the Primroses had spread to any extent; in fact, they seem to have decreased in number. It would be of interest to re-examine the wood to see whether any Primroses still survive, and to examine the edaphic conditions. Another case of interest is that of Knapwell Wood (Fig. 1); an examination in 1937 showed that Oxlips were abundant in the centre of the wood, and that Primroses occurred only round the margins, a situation that suggested that the Primroses had recently invaded the wood or been introduced into it; they were absent from the Oxlip community of the centre of the wood, in which Mercury was abundant (Table 1). On revisiting the wood in 1947, the main communities showed little change, but Primroses appeared to be far less frequent; they were absent along some of the margins, and rare on others. There is thus evidence here that the relative abundance of Oxlip and Primrose may change in the course of ten years, though the cause of the change has not been ascertained.

Christy (1922*a*) suggested that the Primrose in Britain was an 'aggressive' and advancing species, and that the Oxlip was retreating and being ousted by the Primrose. The cases of Peverell's Wood and of Knapwell Wood show that this may not always be true; for here Oxlips have held their ground, and Primroses have certainly not increased. Our investigations lead us to believe that, so far as the species are 'micro-geographically' isolated in woodland, the areas occupied by the species are not changing at present; and the data from Buff Wood suggest that, in woods where both species occur together, competition between them may lead to a balanced condition which is probably of some stability.

III. HYBRIDIZATION IN BUFF WOOD

In woods in which both Primrose and Oxlip occur, interspecific hybridization takes place; the hybrid swarms which are produced are well known. The flowering season of the Oxlip is fairly short; flowering is usually at its maximum towards the end of April. The flowering season of the Primrose begins earlier and ends somewhat later than that of the Oxlip. The kind of insect responsible for cross-pollination is not known with certainty; the matter is discussed in detail by Christy (1922*c*). We have frequently observed both hive-bees and humble-bees visiting the flowers of Oxlips and Oxlip-Primrose hybrids.

When Christy advanced the hypothesis that the Oxlip is, in Britain, a 'retreating' species, he suggested that one of the reasons for its retreat was that it was being 'hybridized out of existence' by the Primrose. This suggestion was based to some extent on the mistaken idea that the Oxlip-Primrose hybrid is sterile; as has been shown by several workers (Valentine, 1947), this is not so. Christy's argument is that, when the two species are in contact, hybrids are formed in the Oxlip population and compete with them, eventually eliminating them; the hybrids in their turn die out, and are replaced by Primroses. It would seem that Christy did not consider it likely that the Oxlip might regenerate by seed and compete successfully with the hybrids; nor, apparently, did he consider the possibility of hybrids being formed in the Primrose population.

Table 5. *Estimated frequencies of Primulas in transects nos.* 1 *and* 2, *expressed as 'points' per unit of transect*

	Primrose areas	Oxlip areas
Primrose	1·22	0·035
Oxlip	0·07	2·03
Hybrids	0·185	0·65

We have attempted to tackle the problem by means of an investigation in Buff Wood. The transects which were laid down so as to traverse the Primrose and Oxlip populations have already been described; and the data for the species and hybrids are given in Figs. 2 and 3. As has been pointed out, the transects show well the sharp boundary between the populations of the two species; and they also show that within their respective areas, Oxlips are more abundant than Primroses. This is probably true in general; it is hinted at by Christy (1897), when he emphasizes the great abundance of the Oxlip in most of its habitats, but he makes no comparative statement about the Primrose. A numerical estimate of relative frequency may be obtained from the transects, as follows; the Primrose data are taken as an example. The number of occurrences of Primrose in Primrose and Oxlip areas is counted and these figures are weighted by scoring 'rare' as 1, 'occasional' as 2, 'frequent' as 3 and 'abundant' as 4; thus 10 rares and 7 occasionals score 24 'points'. The number of 'points' per unit of transect, taking the 4 × 2 ft. section of the transect as the unit, is then worked out. Thus, in transect 1 (Fig. 2), Primrose has $76\frac{1}{2}$ 'points' in 70 units of transect, and in transect 2 (Fig. 3), $74\frac{1}{2}$ 'points' in 54 units of transect. Summing these, we have 151 'points' in 124 units, or 1·22 'points' per unit of transect. A similar calculation for Oxlips and hybrids gives the figures in Table 5.

Figs. 2 and 3 and Table 5 also show that hybrids are much more frequent in the Oxlip area than in the Primrose area. In assessing these data the question of the identification of the hybrids must arise. Artificial F_1 hybrids have been described (Valentine, 1947), but F_2 and backcross hybrids have as yet only been reared to the seedling stage. The latter cannot therefore be identified with certainty in the field; Heslop-Harrison (1931) has stated that backcrosses to Primrose are with difficulty distinguished from pure Primroses; and it is probable that some F_2 types may closely resemble F_1 hybrids. In the transect records the method used was to score as Oxlip or Primrose only such plants as seemed, by careful inspection of all their visible characters, to belong to the pure species; as the great majority of the plants were flowering, this could be done with some confidence; all other plants were recorded as hybrids. In only about 5% of the individual areas, of which the transects were composed, was any doubt felt about the status of the plants examined.

A further check on identification is provided by data collected in 1947, when two plots, each 5 by 2 ft., in the Oxlip area of Buff Wood, were carefully examined. The Primulas growing in the plots are listed in Table 6. What appeared to be clones were scored as single plants, though cloning was not confirmed by tracing the rhizomes. The plots are by no means typical of the Oxlip area as a whole; thus no. 1 contains an abnormally high proportion of hybrids, and no. 2 a quite exceptional number of Primroses. The selection of these plots was deliberate, in order that Oxlips from areas of high hybrid frequency might be examined.

Table 6. *List of Primulas growing in two selected plots, Oxlip area of Buff Wood*

	Plot 1	Plot 2
Flowering plants of Oxlip	16	3
Flowering plants of Primrose	0	7
Flowering plants of F_1 Hybrid	13	6
Flowering plants of putative F_2 and backcross hybrids	2	4
Plants not in flower	9	3

Table 7. *Pollen of Oxlip and* F_1 *hybrids, Buff Wood*

Percentage of bad pollen	0–5	6–10	11–15	16–20	20–65
No. of Oxlips	11	3	3	2	0
No. of F_1 hybrids	0	3	6	3	6

The nineteen plants identified as Oxlips had all the normal Oxlip characters; a typical flower from each plant was measured (using length of calyx and calyx-tooth, breadth of petal and diameter of corolla). The measurements fall within the range normal for Oxlip, as determined both from our own data and from those of Fey (1927) for Central European plants. The pollen of the plants was also examined in acetocarmine, and for each plant 200 grains were counted. A certain amount of bad pollen (shrunken, empty grains) is commonly found in the Oxlip, though the proportion does not usually exceed 15%. Figures for the nineteen Oxlip plants in the plots are given in Table 7.

Data for the F_1 hybrids are given for comparison; the wide range that they show is paralleled by that in the small number of artificial hybrids which have so far been examined. It is clear that the data do not permit a distinction between Oxlips and hybrids on the basis of pollen alone, but they support the conclusion that most of the plants identified as Oxlips in the field are correctly so identified. The data of Table 6 also confirm the opinion formed, when plotting the transects, that the majority of the hybrids are F_1 hybrids, although, as has been pointed out, this cannot be stated with certainty. It is worth noting here that Christy (1922*a*) states that he is 'not conscious of ever having seen a plant which appeared to be a second-cross hybrid'.

Discussion of results

Two main factors affect the establishment and maintenance of hybrids in an Oxlip-Primrose population: the first is seed-setting and germination; and the second, competition. The first factor has been dealt with in a previous paper (Valentine, 1947), where it was shown that the seed set was always worse in interspecific than in intraspecific crosses, the type of hybrid seed differing in the reciprocal crosses. Results obtained in the years 1937–40 showed about 10% germination of hybrid seed with Oxlip as female parent, and a similar figure was obtained, with different stocks, in 1946–7. When Primrose was the female parent of the seed, however, the earlier results gave only 0·1% germination (a single seedling), while the recent seeds gave about 20% germination; the seeds, both in range and type, were similar in both periods. This difference is attributable either to a difference in the stocks used (as suggested in Valentine, 1947), or to the conditions of germination; the conditions were as comparable as was possible; the seeds were sown and kept over-winter in cold frames. The great rigour of the winter of 1946–7, and the 3 months exposure of the seeds, in soil, to a temperature of 0° C. (under snow) may, however, have affected germination, and we now incline to the view that this is the explanation of the difference in the two sets of results; experiments to test the hypothesis are planned. If it be correct, then in most years very few germinable hybrid seeds would be formed as a result of interspecific crosses in the Primrose area, while a significantly larger number would be formed in the Oxlip area; only after very rigorous winters would the numbers be approximately the same. The hypothesis clearly is one which best fits the facts described, namely, the much greater frequency of hybrids in the Oxlip area.

The second factor is that of competition, and the effect of this is much more difficult to assess correctly. Hybrid seedlings will spring up alongside greater numbers of seedlings of the pure species, for the seeds of the pure species are formed in greater numbers and have a much higher germination. Competition will occur at all stages from seedling to adult; and its effect will be complicated by the conditions in the wood. Much more space may be available on the woodland floor immediately after coppicing than under a 15-year growth of coppice; and possibly insect visitors are more frequent and seed-production greater too when the light intensity is higher. There may thus well be a cycle in the woodland, hybrids becoming established early in the coppicing cycle and dying out again as the coppice grows up. The final result will depend on a complex of factors, of which the way the wood is managed is clearly one. Thus it is conceivable that the rate of hybrid formation (seed-setting, germination, etc.) might be greater, say in the Primrose than in the Oxlip area, but that the final position was one in which hybrids were commonest in the Oxlip area.

Let us now examine the data in the light of these considerations. In the Oxlip area of Buff Wood there is a population consisting mainly of Oxlips and hybrids, and Primroses are rare. We conclude that hybrids resemble Oxlips sufficiently in their ecological requirements to be able to survive and compete with them, but that Oxlips retain certain ecological advantages which enable them to maintain themselves as such in considerable numbers, i.e. they are not swamped by the hybrids (cf. the figures for relative abundance in Table 5). A high proportion of the hybrids present appears to be F_1 hybrids; in view of the relative abundance of the types involved, backcrosses might be expected to be more frequent in the second and subsequent generations than F_2, F_3 hybrids, etc., but data to determine the proportion of second generation and subsequent hybrids are not yet available; a certain proportion of both F_2 and backcross types is to be expected. Migration of Primrose into the Oxlip area appears to be mainly as pollen and only rarely as seed.

In the Primrose area there is a population consisting mainly of Primroses; the frequency of hybrids is much less than in the Oxlip area. There is also a small number of Oxlips (Table 5). In view of the uncertainties as to the germination of F_1 hybrid seed it is not possible to be sure of the reasons for the low hybrid frequency, but low germination is probably an important factor. The possibility cannot be overlooked, however, that the hybrids find the conditions of the environment in the Primrose area less favourable than in the Oxlip area, and that, therefore, even when they are formed, they do not survive in large numbers. Most of the hybrids in the Primrose area appear to be F_1 hybrids; it is possible to distinguish putative backcross hybrids by eye, but their pollen is not significantly less fertile than that of pure Primroses, and, until more experimental evidence is forthcoming, their frequency cannot be assessed; it is probably very low. Migration of Oxlip into the Primrose area is thus effectively small, whether as pollen or as seed.

In the ecological discussion (in which the hybrids were neglected) it was concluded (p. 120) that the relation between Oxlip and Primrose in the wood might be one of neither advance nor retreat, but of approximate stability. The question arises as to whether the proportion of hybrids at present observed in the population is also stable. The situation in Buff Wood may be formulated:

$$\text{Oxlips} + \text{Hybrids} \parallel \text{Primroses} + \text{Hybrids}.$$

On Christy's hypothesis,

$$\text{Oxlips} + \text{Hybrids} \rightarrow \text{Hybrids} \rightarrow \text{Primroses};$$

but, as has already been pointed out, Christy does not state clearly the supporting evidence. It is possible that he observed that in mixed populations hybrids were more frequent among Oxlips than among Primroses; this has been demonstrated above for Buff Wood, and may well be generally true; but it does not follow that replacement is occurring. We have shown how data from seed-setting and germination in reciprocal crosses provide a possible reason for the greater frequency of hybrids among the Oxlips; and the fact that most hybrids are apparently of the F_1 generation may be interpreted to mean that the composition of the population is not varying greatly. Valentine (1947) has shown that the F_1 offspring of the cross vary greatly in vigour; if only the more vigorous survive, and produce comparatively few and less vigorous offspring in subsequent generations, then a steady state might soon arise, in which new F_1 hybrids became established at about the same rate as the old ones died.

The question may be approached by considering the possible age of the mixed population; obviously, if one of the Primula species reached the wood only very recently, a condition of stability would hardly have been attained. We shall, therefore, consider very briefly here the past history of the woodland.

The fact that Oxlips are the only woodland Primulas found in the central parts of the boulder clay plateaux indicates that they colonized these regions at a time when Primroses were not present at all, or were prevented from invading by a barrier of some kind, or were originally present but were ousted by the Oxlips. After colonization, the area occupied by the Oxlips may have fluctuated from time to time as, for example, climate fluctuated; but the arrival of man will have eventually introduced important new factors. The woodland cover, probably much more extensive than at present, will have been broken up and much of the cleared land drained and cultivated. Habitats suitable for the Oxlip would thus become fewer, and it would tend to retreat into the few remaining woodlands, where conditions of poor drainage persisted. If it be assumed that the climate at the time at which these events took place was much the same as it is now, the woods which survived would be effectively isolated from one another in respect of the migration of Primroses, as they are now. Thus the present distribution of Primroses round the edge of the Oxlip regions would be approximately the same as at the time when the region was cleared and cultivated; the minimum age of the mixed populations could thus be fixed. If, on the other hand, the climate at the time of the clearance was such as to permit migration of Primroses across unwooded country, i.e. was more oceanic than at present, then such migration may have continued until the climate veered towards the more continental conditions of the present time; and the estimate of the minimum age of the mixed populations would be decreased. Data cited by von Post (1946) suggest that minor climatic fluctuations of the type suggested here may be more frequent and have more effect on vegetation than is generally imagined.

We cannot at present decide which course events followed. As regards clearance and drainage there is evidence that the Oxlip regions were settled by man in early medieval times (Darby, in Godwin, 1938). As regards Buff Wood itself there is a map extant, dated 1750, which shows the wood in almost exactly its present shape. A minimum age of 200 years for the mixed population is thus probable, and the actual age may, of course, be much greater. Our conclusion as to the stability of the Oxlip and Primrose areas in the wood must also mean, if it is correct, that the mixed population is fairly old; for it will have taken a considerable time for the species to settle down into 'ecological equilibrium'.

It will be clear that conclusive evidence as to changes that may be occurring in the Primula population in Buff Wood cannot yet be provided; at present we can do no more than offer hypotheses. These are, first, that the mixed population is a fairly old one, sufficiently old for the species to have come into 'ecological equilibrium'; and secondly, that the proportion of hybrids present is not changing appreciably. Hybridization is thus symptomatic of the mixture of the species, but is not fundamentally concerned in the replacement of one species by the other. Such replacement may eventually occur, but it will be due to climatic or edaphic changes, as suggested earlier (p. 120); thus, in one wood, Primrose might tend to increase at the expense of Oxlip, in another the reverse process might occur. We do not consider that there is any general tendency towards movement in one direction only, viz., Oxlip → Primrose, as suggested by Christy; but more evidence is needed. This can be provided by autecological investigations, by transplant experiments, by further breeding experiments, and by comparative studies of more woods at the margin of the Oxlip regions.

IV. EXPERIMENTAL TAXONOMY

We conclude with a brief discussion of the status of the Primrose and Oxlip as species; from the viewpoint of experimental taxonomy the data presented here are relevant.

Primrose and Oxlip are morphologically very distinct; the characters which are given in Valentine (1947) concern leaf, peduncle, calyx, corolla, fruit and seed, and indumentum, and the plants breed true for these characters. The species differ physiologically, for example, in extent of flowering time, and in times of production and duration of leaves; and the differences in geographical distribution and in ecology have been pointed out. For orthodox taxonomy these differences are quite sufficient to justify the separation of Primrose and Oxlip as well-marked species; but in the classification of experimental taxonomy, or biosystematics as it is sometimes called, a genetical criterion is used, of which the most recent account is given by Clausen, Keck & Hiesey (1945). These authors distinguish four biosystematic units, the names of which are taken over from Turesson and Danser, though the meanings of some of them are modified; they are, the ecotype, the ecospecies, the coenospecies and the comparium. It is with the first two of these that we are concerned here.

Ecotypes are defined as genetically and physiologically distinct ecologic races (geographical races are also included in the definition). Ecotypes, which are components of a species, are suited to a series of contrasted environments; and there is no genetic obstacle to a free interchange of their genes when they meet and hybridize. The criterion of the separation between ecotypes is ecological or geographical, but not genetical isolation; the morphological criterion is relegated to an insignificant position; and the statement is made that 'some so-called species, not separated by genetic barriers but recognized only by morphology and ecology are but ecotypes of one species'. We shall return to this statement.

Ecospecies are said to approximate to the conventional species of the orthodox taxonomist; they are defined in terms of a larger composite unit, the coenospecies, thus: species capable of a limited exchange of genes with one another are ecospecies of one coenospecies. The definition is expressed in another way by the statement that the genes of related ecospecies cannot be freely interchanged without seriously impairing the ensuing development of the offspring. There is thus a positive side to the definition, namely, the possibility of limited exchange of genes; and a negative side, namely, impairment of the offspring by exchange of genes. It will be seen that in this definition the criterion of separation is partial genetic isolation; other criteria may be implicit, but they are not stated.

The application of these terms to the species pair Primrose-Oxlip is fairly clear. The species can certainly exchange genes; the exchange is limited, in the first place, by the phenomena that occur in the setting of hybrid seed, which have been previously described (Valentine, 1947); in addition, many of the hybrid combinations are inviable, and of the F_1 plants which survive, a certain proportion is dwarf. Further, results from reciprocal crosses are not the same; the proportion of seeds with embryos, and the size of seed, both differ, probably as a result of differences in the embryo-endosperm balance. The F_1 plants are fairly fertile, but not so fertile as the parents; the fertility of the F_2 generation has yet to be determined. These facts all indicate that Primrose and Oxlip are ecospecies of a coenospecies.

It is of interest to compare this case with others. Following the extensive investigations of Gregor and his collaborators on the sea plantains (*Plantago maritima* L. and its allies), the cytology of certain members of the group has been explored by Earnshaw (1942). Earnshaw finds that the sea plantains of the European Alpine region, though closely resembling one another morphologically, are of two kinds: diploid ($2x=12$) and tetraploid ($2x=24$). Diploid and tetraploid populations are highly stable and intrafertile, but markedly intersterile; a very limited amount of gene exchange between the two groups, for example, by the production of fertile hybrids as a result of the successful functioning of diploid gametes, is possible under experimental conditions, though it has not been demonstrated in the field. The diploid and tetraploid populations are therefore correctly regarded as ecospecies of a coenospecies. The differences between these and the *Primula* ecospecies are clear; in the one case there are wide differences in morphology, ecology and geographical distribution and a relatively weak barrier to interbreeding; in the other, a strong barrier to interbreeding, but only slight morphological and ecogeographical differences. These contrasting kinds of ecospecies are probably the most typical; another example of the *Primula* type is provided by *Festuca pratensis* and *Lolium perenne*, described by Jenkin (1933); in the formation of F_1 seed in reciprocal crosses, this pair shows resemblances to the *Primula* species, as has been pointed out by Valentine (1947), and the morphological differences between the *Festuca* and the *Lolium* are considerable; but there is a difference from the *Primula* pair in the much greater infertility of the hybrid, which is, nevertheless, sufficiently fertile to cause Clausen *et al.* (1945) to classify the *Festuca* and the *Lolium* as ecospecies. Another example of the *Plantago* type is provided by *Viola riviniana* and *V. reichenbachiana*; the former is tetraploid with $2n$ usually $=40$ (Valentine, 1941), the latter diploid. The species are very similar morphologically and cover roughly the same geographical area, though differing somewhat in their ecology; the hybrid between them is triploid and highly infertile, but it produces a few seeds when selfed or in backcrosses (Valentine, unpublished data), so that the species must be regarded as ecospecies.

The term ecospecies thus covers a heterogeneous assemblage of groups; this is not unexpected when it is remembered that the category is based on a single criterion only, namely, that of genetic isolation. The limitations of the classification are well illustrated by Turrill's exposition (1946) of the case of *Silene maritima* and *S. cucubalus*, which he and Marsden-Jones have so thoroughly investigated. These are perfectly well-defined taxonomic species, differing markedly in morphology and ecology, rarely hybridizing in the field and when doing so, forming only very limited hybrid populations; yet experiments demonstrate that the species (or at least, British representatives) are perfectly interfertile—there is no barrier to gene exchange. They have thus to be classed as ecotypes—a conclusion which the orthodox taxonomist is unwilling to accept. It is unfortunate, too, that the term ecotype, coined originally by Turesson (1922) to denote ecological races, differing markedly in ecology but only slightly in morphological characters, should have been so modified that its original and most useful meaning is in danger of being obscured. The changes in the meaning of the term since 1922 are discussed by both Gregor (1944) and Turrill (1946); Turrill concludes that the terms ecotype, ecospecies, etc., are useful in their limited field, but that this field is much less wide in scope than that of taxonomy proper, which has to take account of criteria other than genetical, and that the terminology of experimental taxonomy should be kept strictly separate from that of orthodox taxonomy. While we agree with this view, we would deplore any tendencies to limit the scope of experimental taxonomy by its terminology. Experimental taxonomy, in our view, should aim at as wide a synthesis as possible of all the data relevant to the classification of specific units; it must attempt to arrive at an understanding of evolutionary mechanisms, using the methods of genetics and cytology, but it must not be divorced from a study of the environment, and the analysis of ecological and geographical factors. A well-balanced approach to these problems is that of Huxley (1942), who distinguishes, *inter alia*, species whose origin is abrupt from those in which it is gradual. Thus, the *Primulas* and the *Silenes*, described above, probably began as ecologic or geographic races of a single diploid species; their differentiation into species has been gradual and probably slow, and has been accompanied by the development of considerable morphological differences; genetical barriers have not yet developed between the *Silenes*, and have developed to a certain stage between the *Primulas*; the *Festuca-Lolium* pair may represent a later stage, in which the hybrid between the species is highly infertile. In these cases, the species have arisen gradually as a result of ecogeographical isolation, and genetical isolation has followed. In the *Plantagos* and *Violas* described above, on the other hand, it would seem likely that the origin of the new types was abrupt, in the *Plantagos* probably by autotetraploidy, in the *Violas* probably by allotetraploidy. The new species resemble the parents closely, but are at once separated from them by an efficient genetical barrier; development of the new species can thus proceed, provided they are able to compete with the parents, and exploration of new ecological and geographical niches is a subsequent process.

It would be useful to distinguish between these types of ecospecies; we suggest tentatively the terms abrupt or *a*-ecospecies, and gradual or *g*-ecospecies. The former type will generally include the polyploids, and also other species whose main cause of origin has been an abrupt change, e.g. a reciprocal translocation involving change in chromosome number, as in some of the *Crepis* species described by Babcock (1947). A single coenospecies may, of course, contain both types of ecospecies.

Distinction between ecotypes and some types of *g*-ecospecies may be difficult, as it is probable that, in some cases at least, ecotypes represent early stages in the formation of *g*-ecospecies, and we are therefore bound to find intermediate forms; the matter is discussed by Dobzhansky (1941). Nevertheless, some modification of the definition of ecospecies seems to be desirable. The experimental taxonomist may be concerned mainly with genetic isolation, but if he is to be realistic it is genetic isolation in nature rather than under experimental conditions which is of evolutionary importance. As pointed out by Dobzhansky (1941), the question to be answered is not necessarily whether certain species are *capable* of free exchange of genes, but whether in fact they do exchange them in the field. The capacity for gene exchange is doubtless important, as future events may bring together species hitherto separated; but the classification should surely take account as well of things as they are.

We therefore propose the following new definition of ecospecies: species are ecospecies of a coenospecies when exchange of genes can take place between them, but when the exchange under either natural or artificial conditions is limited in some way. This definition covers the case of the species of *Silene*, mentioned above; because of the limited gene-exchange between them in the field, they rank as *g*-ecospecies, not ecotypes. In the hierarchy of *g*-ecospecies, *Silene maritima* and *S. cucubalus* thus take their place at a level at which ecogeographical factors are the important isolating factors. *Festuca pratensis* and

Lolium perenne may be regarded as standing at the other end of the scale, for with these species ecogeographical isolation is no longer effective, and the isolating factors are genetical. Primrose and Oxlip occupy an intermediate position; for both ecogeographical and genetical factors are important in maintaining the isolation of the species and their survival as distinct units.

SUMMARY

1. The geographical and ecological distribution of the Primrose and the Oxlip are briefly described. A revised map of their distribution in the Oxlip region to the west of Cambridge is given, and it is pointed out that the woods are 'micro-geographically' isolated from one another in respect of Primrose and Oxlip.

2. A description is given of the flora and the environmental conditions in a Cambridgeshire wood which contains both Primrose and Oxlip. The species occupy distinct areas in the wood, and it is shown that these are similar floristically, but can be distinguished by the greater abundance of *Filipendula ulmaria* in the Oxlip area; it is considered that this difference is correlated with a difference in soil water relations and that Oxlip and Primrose may be in a condition of ecological equilibrium, neither extending its area at the expense of the other.

3. It is shown that in the wood examined interspecific hybrids (probably mostly F_1 hybrids), are much more frequent in the Oxlip area than in the Primrose area, but that they are outnumbered by the pure species in both areas. The relative viability of the seeds from reciprocal crosses, and competition, are the main factors which affect this situation.

4. It is thought likely that the *Primula* population is of considerable age, and that the proportion of hybrids which it contains is approximately stable. Christy's hypothesis, that the Oxlip is being hybridized out of existence, is not supported, and it is suggested that the fundamental factors which govern the proportions of Oxlips and Primroses in the population are climatic and edaphic.

5. The experimental taxonomy of the Primrose and Oxlip is discussed, and it is concluded that they are ecospecies of a coenospecies. Different types of ecospecies are reviewed; a revised definition of the term ecospecies is suggested, and it is proposed that two types of ecospecies, those whose origin was abrupt and those whose origin was gradual, be distinguished.

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REFERENCES

Adamson, R. S. (1910). Note on the relationships of *Primula elatior* and *P. vulgaris* to soil conditions. *Trans. Bot. Soc. Edinb.* **24**(2), 84.

Adamson, R. S. (1912). An ecological study of a Cambridgeshire woodland. *J. Linn. Soc.* (*Bot.*), **40**, 339.

Babcock, E. B. (1947). Cytogenetics and speciation in *Crepis*. *Advances in Genetics*, **1**, 69.

Christy, M. (1897). *Primula elatior* in Britain. Its distribution, peculiarities, hybrids and allies. *J. Linn. Soc.* **33**, 172.

Christy, M. (1922*a*). *Primula elatior* Jacquin: its distribution in Britain. *J. Ecol.* **10**, 200.

Christy, M. (1922*b*). The origin of the hybrid *Primula elatior* × *vulgaris* demonstrated experimentally in the field, with notes on other British *Primula* hybrids. *New Phytol.* **21**, 293.

Christy, M. (1922*c*). The pollination of the British Primulas. *J. Linn. Soc.* (*Bot.*), **46**, 105.

CHRISTY, M. (1924). *Primula elatior* Jacquin: its distribution in Britain. Supplementary note. *J. Ecol.* **12**, 314.

CLAUSEN, J., KECK, D. D. & HIESEY, W. M. (1945). Experimental studies on the nature of species. II. Plant evolution through amphiploidy and autoploidy, with examples from the *Madiinae*. *Publ. Carneg. Instn.* No. 564.

CORBIÈRE (1894). *Nouvelle Flore de Normandie.* Caen and Paris.

DOBZHANSKY, T. (1941). *Genetics and the Origin of Species.* New York.

DRUDE, O. (1912). The Flora of Great Britain compared with that of Central Europe. *New Phytol.* **11**, 236.

EARNSHAW, F. (1942). Experimental Taxonomy. V. Cytological studies on sea plantains allied to *Plantago maritima* L. *New Phytol.* **41**, 151.

FEY, L. (1927). Untersuchungen zur Phänanalyse des Artbastardes *Primula variabilis* Goupil, der Eltenarten, und von *Primula elatior* (L.) Schreber. *Arch. Klaus-Stift. Vererb-Forsch.* **3**, 299.

GAUSE, G. F. (1934). *The Struggle for Existence.* Baltimore.

GODWIN, H. (1938). *The Botany of Cambridgeshire.* Ch. IV, 'Scientific survey of the Cambridge district'. London.

GOOD, R. D. (1944). On the distribution of the primrose in a southern county. *Naturalist, Lond.*, **41**.

GREGOR, J. W. (1944). The ecotype. *Biol. Rev.* **19**, 20.

HEGI, G. (1931). *Illustrierte Flora von Mittel-Europa.* Band V (III). Munich.

HESLOP-HARRISON, J. W. (1931). The Northumberland and Durham Primulas of the section *Vernales*. *Trans. North. Nat. Un.*, **1**, 48.

HUXLEY, J. S. (1942). *Evolution: the Modern Synthesis.* London.

JENKIN, T. J. (1933). Interspecific and intergeneric hybrids in herbage grasses. Initial crosses. *J. Genet.* **28**, 205.

LITTLE, J. E. (1935). The Ivel district of Bedfordshire. *B.E.C. Rep.* **11**, 50.

PAX, F. & KNUTH, R. (1905). *Das Pflanzenreich. IV. Primulaceae.* Leipzig.

ROUY, G. (1913). *Flore de France.* Asnières, Rochefort and Paris.

SALISBURY, E. J. (1920). The significance of the calcicolous habit. *J. Ecol.* **8**, 202.

TURESSON, G. (1922). The species and variety as ecological units. *Hereditas*, **3**, 100.

TURRILL, W. B. (1946). The ecotype concept. A consideration, with appreciation and criticism, of recent trends. *New Phytol.* **45**, 34.

VALENTINE, D. H. (1941). Variation in *Viola Riviniana* Rchb. *New Phytol.* **40**, 189.

VALENTINE, D. H. (1947). Studies in British Primulas. I. Hybridization between Primrose and Oxlip (*Primula vulgaris* Huds. and *P. elatior* Schreb.). *New Phytol.* **46**, 229.

VON POST, L. (1946). The prospect for pollen analysis in the study of the earth's climatic history. *New Phytol.* **45**, 193.

WATT, A. S. (1938). *The climate of Cambridgeshire.* Ch. III, 'Scientific survey of the Cambridge district'. London.

THE ECOTYPES OF *MELANDRIUM DIOICUM* (L. EMEND.) COSS. & GERM.

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(With Plate 3 and 1 figure in the text)

CONTENTS

	PAGE
I. Introduction	131
II. British Material	132
(1) Historical	132
(2) Original observations	132
(*a*) Lowland ecotype	132
(*b*) Coastal and sub-alpine ecotypes	133
III. Discussion of the history and distribution of the ecotypes	139
(1) Modes of origin of ecotypes	139
(2) Distribution of the ecotypes	139
(3) Historical factors in distribution	140
(4) Necessity for further investigation	143
Summary	143
References	144

I. INTRODUCTION

Turesson (1922) first investigated the ecological significance of variation within the species *Melandrium dioicum* (under the name *M. rubrum* Garcke). He recorded a difference between a hereditarily thick-leaved type from the sea cliffs of Kullen in north-west Scania and a thin-leaved type from the inland beech-woods of the same province. The thickness of the ground-leaves of the former (determined in autumn), after 2 years' culture in the experimental garden, was 575–615 μ, while the corresponding values for the type from the beech-woods were 295–350 μ. Although Turesson maintained that the difference between the two types was constant, the next report extended the ranges to 425–615 μ for the coastal type and 300–450 μ for the beech-wood type, showing some overlapping. The coastal type was also reported from further stations on the east and west coasts of Scania and was stated to flower a fortnight earlier than the thin-leaved type.

A sub-alpine type was claimed (Turesson, 1925, p. 186) with even thicker leaves, which were reported to have reached thicknesses of 500–680 μ in culture. This type was transplanted to Akarp from northern stations in Sweden (Luleå, Abisko, Ornskoldsvik, Jamtland). The ground-leaf rosette attained greater dimensions in the course of the vegetative period and the flowering period fell somewhat earlier than that of the southern coastal type. These northern plants appeared to correspond with plants from sub-alpine stations in the Alps and Carpathians, which were also brought into culture.

A further type was described, limited, in this case, to alpine regions. This was represented in the cultures by material from Finse (Norway), where the specimens were collected at 1450 m. above sea-level in lichen heath. This type possessed leaves whose thickness sometimes attained 750μ in culture. The rosette formation was still more marked, here, than in the other types, but an outstanding feature was the dwarf habitus of this type, which was partially maintained in culture.

Turesson (1925, p. 225) grouped his material according to the following scheme:

Oecotypus salinus: *Melandrium rubrum* (= var. *crassifolium* Fr.).
Oecotypus alpinus: *M. rubrum* (= var. *subacaulis* Norm. (partly)).
Oecotypus subalpinus: *M. rubrum* (= subsp. *lapponicum* Simm. (partly));

in addition to the thin-leaved lowland (beech-wood) type.

II. BRITISH MATERIAL

(1) *Historical*

In 1927 Turesson reported upon investigations of the osmotic and suction pressures of the epidermal cells of plants from Sussex and compared them with similar observations on materials from the Faeroes, Kalmar (on the south-east coast of Sweden) and Stehag (an inland locality in southern Sweden). The Kalmar plants were thick leaved while the others were thin leaved, although the Faeroe plants were of a distinctly hairy type. The Sussex plants were stated to be of an inland, woodland type, with leaves even thinner than those of the Swedish beech-wood type and almost glabrous. His conclusions were that the woodland ecotypes from Sussex and Stehag agreed in possessing high *osmotic* pressures, while the maritime Faeroe and Kalmar plants had low osmotic pressures. Despite this, however, the Sussex and Faeroe plants agreed in showing low *suction* pressures, while those of the Stehag and Kalmar plants were higher. This latter observation he explained by the fact that the Stehag and Kalmar plants came from a region of lower rainfall, while the Sussex and Faeroe plants both originated from a region of higher rainfall.

Another paper (Turesson, 1928) is concerned with the transpiration rates from plants of the lowland ecotype from a beech-wood at Stehag and an abundant maritime type from southern England. The present author has no knowledge of the latter type. Turesson found that the English material had a lower transpiration rate per sq.cm. of leaf surface and a correspondingly higher osmotic pressure than the Stehag plants. This he correlated with the more difficult conditions met with by the former. It has occurred to the present author that any material of *M. dioicum* growing in exposed places in southern England is very likely to be contaminated by hybridization with *M. album* (see Baker, 1945*a*, *b*, 1946, 1947*a*, *b*), and Turesson may have been measuring the effect of introducing genes from *M. album*, a species which can certainly exist on cliff tops and shingle beaches.

(2) *Original observations*

(*a*) *Lowland ecotype*

The red campion which is typical in the British Isles is a woodland mesophyte manifestly unable to compete successfully in exposed habitats (Baker, *loc. cit.*). Plants were grown in an experimental garden set up at Sunbury-on-Thames, Middlesex, from seed which was obtained from Broadwater Wood, Walton-on-Thames, Surrey. These plants showed an average thickness of 261μ (S.D. ± 57) for the ground-leaves when measured in autumn. Similar figures were given by leaves taken from plants growing in nature as long as hybridization with *M. album* had not occurred. The thickness of the leaves is slightly influenced by the environment, for plants of this ecotype from a willow-wood at Wraysbury, Buckinghamshire, showed a difference of about 5·4% in the thickness of leaves developed upon plants growing in the uncut wood (relative light intensity 29·6–100%, mostly nearer the former) and mature plants growing in a portion of the wood which had been cut 4 months previously (June 1943). Similarly, the average leaf thickness of the plants growing in the experimental garden at Sunbury was 7·6% higher than that of the parent population in Broadwater Wood, Walton-on-Thames.

(*b*) *Coastal and sub-alpine ecotypes* (*Oecotypi salinus et subalpinus*)

These ecotypes are difficult to distinguish from each other as the descriptions given by Turesson (1925, p. 185) are not very precise.. Careful search of some British herbarium material has shown that there are specimens with the appearance of one or other of these ecotypes from the stations shown in Table 1.

Table 1

Location	Collector	Herbarium
Shetland Isles:		
Burrafirth, Unst	R. Tate	Cambridge University
Burrafirth, Unst	F. J. Hanbury	Cambridge University
Burrafirth, Unst (2 specs.)	W. H. Beeby	S. London Bot. Inst.
Northmaren	W. H. Beeby	S. London Bot. Inst.
Mid Yell Voe	W. H. Beeby	S. London Bot. Inst.
Tetlar, Mid Yell	W. Brown	S. London Bot. Inst.
Noup of Noss (2 specs.)	W. R. Price	Roy. Bot. Gard. Kew
Foula	J. Gladstone	Roy. Bot. Gard. Kew
Burrafirth, Unst	W. H. Beeby	British Museum (N.H.)
Noup of Noss	W. E. & H. Smith	British Museum (N.H.)
Green Holm, Scalloway (2 specs.)	E. Straker	British Museum (N.H.)
Burrafirth, Unst	W. A. Shoolbred	Nat Mus. Wales, Cardiff
Scotland:		
Lochnagar, Braemar	A. Croall	Cambridge University
N. of Loch Laggan, Inverness	E. S. Marshall	Cambridge University
Haddington	R. Memertzhagen	British Museum (N.H.)
Ben Lawers	J. D. Hooker(?)	Roy. Bot. Gard. Kew

The records from the Shetland Isles, at least, are referable to '*Melandryum dioicum* var. *zetlandicum*'. This form was described by Compton (1920) from material in the herbaria of the University of Cambridge, Royal Botanic Gardens (Kew), British Museum (Natural History) and the South London Botanical Institute. His description is as follows:

'Stem simple, very stout, erect, densely hairy. Petioles of basal leaves narrow, broadening very suddenly to the laminae. Laminae of basal leaves broadly elliptical: of stem leaves broadly ovate or elliptical, soft and very downy upon both surfaces. Bracts much larger than in var. *villosum*.* Flowers in dense subsessile terminal clusters and in the axils of uppermost leaves, larger than in var. *villosum*. Petals of a deeper purple. Not so far known to breed true.'

An amplified description is given by Baker (1947*a*), where this variety is raised to the status of subspecies.

The material cited by Compton has been studied along with other material from the Shetland Isles which is present in these herbaria and in that of the National Museum of

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* Compton's name for *M. dioicum* subsp. *villosum* (Compt.) Baker (Baker (1947)) (= the lowland ecotype), not to be confused with '*M. silvestre* Roehl. var. *villosum* Celak' (see p. 140).

Wales (see Table 1). In addition, fresh material was received from Miss Rosina Isbester of Leraback, Foula, and comparison of some of the plants with the material used by him showed close agreement. These plants agreed completely with the quoted description, except for petal-colour in some cases (see p. 136). They were presumed, therefore, to be of the same subspecies and were planted in the experimental garden. Seeds were also obtained from these two sources, from plants which answered most closely to Compton's description (which was sent to the two collectors). This seed was sown and produced typical plants of the subspecies.

In the herbarium at Kew there is one of the sheets circulated by Fries in 1865 and labelled by him: '*Melandrium diurnum* v. *crassifolium Fr. Cat. Hort. Ups.* s. *Melandrium maritimum* Fr. inscr.' These plants were grown at Uppsala from seed collected at Christiansund in Norway. The two specimens at Kew are from different plants and there is a considerable difference between them. The left-hand one (staminate) agrees very well with *M. d.* subsp. *zetlandicum*, except that it is taller (over 35 cm. high compared with an average of 23 cm. for herbarium specimens of plants of the latter subspecies from Shetland stations) and slightly less hairy (although still more hairy than *M. d.* subsp. *villosum*). Both of these differences may be the result of growth in a garden. The right-hand plant, which is pistillate, has a much weaker stem and the whole plant is hardly hairier than *M. d.* subsp. *villosum*, this difference between Fries's plants being especially noticeable in the inflorescence where the calyx of the latter plant is covered with short hairs. These contrast with the long hairs of the other specimen and of *M. d.* subsp. *zetlandicum*. There are fewer flowers in the cluster and the capsules are small.

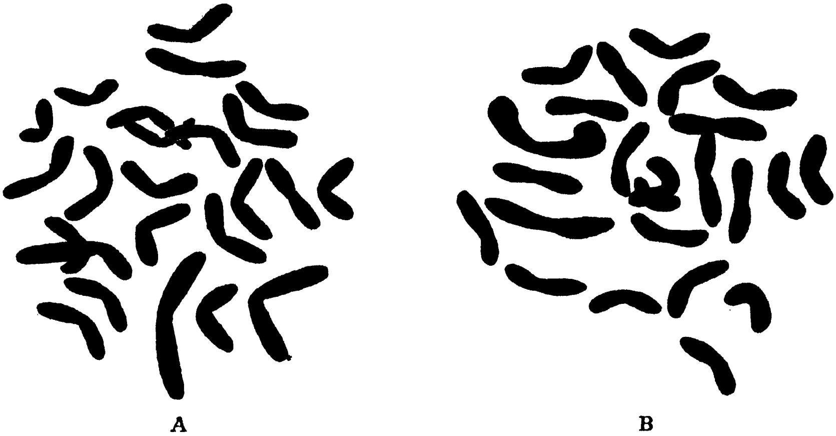

Fig. 1. Somatic metaphase plates from: A, *Melandrium dioicum* subsp. *zetlandicum* (from seed from Foula); B, *M. dioicum* subsp. *villosum* (from seed from Meanwood, Yorkshire). $2n = 24$ in both cases (× 4000). Preparations from material fixed in Belling's modification of Navashin's fluid, sectioned and stained with iron alum-haematoxylin. Drawings made from photographs.

Turesson (1925, p. 189) has stated that specimens circulated by Fries, and bearing this label, are identical with his thick-leaved coastal ecotype. Löve (1942*a*) has examined a plant which she identified as '*M. crassifolium*' and which was a representative of the coastal ecotype. Thus, it would appear that *M. d.* subsp. *zetlandicum* (Compt.) Baker truly represents Turesson's coastal ecotype in this country (see Table 2 and Pl. 1).

Table 2. *Comparison of the Lowland and Coastal Ecotypes of* M. dioicum *grown in the Experimental Garden.* (*See also Pl.* 1)

Except where mentioned, characters appear to correspond in the two ecotypes.

Character	Lowland ecotype (based on material from Surrey)	Coastal ecotype (based on material from the Shetland Isles)
Flowers:		
Diameter of corolla	Usually 1·8–1·9 cm.	Often reaching 2·7 cm.
Coloration of petals	White to rose-madder or magenta	White to magenta
Shape of calyx teeth	Equilaterally triangular	May be slightly wider at base
Shape of inflorescence	Loose dichasial cyme	Tighter. Flowers collected in terminal clusters
Shape of capsule	Spherical, small	Spherical to conical, larger
Seeds:		
Size	*c.* 0·5 × 0·3 mm.	*c.* 1·0 × 0·5 mm.
Weight (of 1000)	*c.* 0·5 g.	1·05–1·6 g.
Stem:		
Diameter	Seldom more than 0·3 cm. Not infrequently branched	Up to 0·6 cm. Seldom branched except in region of inflorescence
Height	50·2 cm. S.D. ± 8·4 in sample	44·1 cm. S.D. ± 7·6 in sample
Hairiness	Generally sparsely hairy	Softly downy
Leaves:		
Shape—Radical	Long petiole tapering from broadly elliptical lamina	Petiole broadening suddenly to more narrowly elliptical lamina
Shape—Cauline	Very broadly ovate. Sessile	Ovate or elliptical, narrower. Sessile
Thickness	Relatively thin (see text)	Often twice as thick (see text)
Hairiness	Generally sparsely hairy	Softly downy
Root system:		
Size	Much divided. Tap-root up to 0·5 cm. in diameter	Coarser. Tap-root up to 2 cm. in diameter
Physiological characters:		
Reaction to exposure and drought	Mesophyte	More xerophytic
Reaction to frost	Rather susceptible (see Åkerlund, 1933)	Probably more susceptible
Tolerance of shade	Considerable	Small

Table 3

Character	1943			1944		
	Fa 1 (♀)	Fa 2 (♀)	Fa 3 (♂)	Fa 1 (♀) (died)	Fa 2 (♀)	Fa 3 (♂)
Length of calyx teeth (mm.)	3	3	2	—	3	2
Average diameter of corolla (cm.)	2·2	2·3	2·1	—	2·4	2·0
Pollen fertility (%)	—	—	—	—	—	75·4
Capsule shape	Globose conical	Globose conical	—	—	Globose conical	—
Weight of 1000 seeds (g.)	1·396	1·375	—	—	1·045	—
Vegetative anthocyanin	None	Plenty	None	—	Plenty	None
Average thickness of ground-leaves (μ)	480	600	310	—	470	330
Average length × breadth of mature ground-leaves (cm.^{2})	30·40	24·84	16·96	—	12·00	9·20
Hairs visible in field of microscope. (Total for both surfaces of leaf.) (Details in Baker, 1946)	5·50	5·20	5·13	—	42·50	14·92
Leaf index $\frac{\text{length of lamina}}{\text{greatest breadth}}$ of stem leaves (5th node)	2·11	3·41	1·66	—	3·00	1·74
Height of plants (cm.)	31	30	25	—	43	30

Of the material received from Foula, two plants (Fa 1 and Fa 2) were certainly of the subsp. *zetlandicum*, while the third plant (Fa 3) did not correspond. Observations made on arrival, and after cultivation for a year in the experimental garden at Sunbury, enabled Table 3 to be drawn up. The examination was restricted in order to avoid damage to the plants, which were required for other investigations. Unfortunately, the cultivation had to be abandoned at the end of the 1944 season.

Apparently, Fa 3 is a representative of *M. d.* subsp. *villosum* var. *albiflorum* (Tin.) Baker, which differs from subsp. *villosum* only in the total lack of anthocyanin in the plant. Fa 1 died during the autumn following transplantation but there seems no doubt that it belonged to the subsp. *zetlandicum*. It was artificially pollinated from *M. album* and *M. dioicum* subsp. *villosum* and in both cases gave viable seed (see p. 138). Thus, both the lowland and coastal ecotypes occur on Foula. The greater number of hairs visible on the same area of the leaves after cultivation may be due to the smaller size of the leaves developed.

The material received from Haroldswick, Unst, confirmed that both ecotypes occur in the Shetlands. Of the three plants, two (U 2 and U 3) were of the coastal ecotype (subsp. *zetlandicum*) while the other (U 1) was of the lowland ecotype (Table 4).

Table 4

Character	U 1 (♀)	U 2 (♂)	U 3 (♂)
Length of calyx teeth (mm.)	3·5	2·5	2·0
Average diameter of corolla (cm.)	1·9	2·2	2·3
Pollen fertility (%)	—	95·2	88·0
Capsule shape	Globose	—	—
Weight of 1000 seeds (g.)	0·30	—	—
Vegetative anthocyanin	Plenty	Plenty	Plenty
Average thickness of ground-leaves (μ)	250	420	410
Average length × breadth of mature ground-leaves (cm.^{2})	11·07	17·88	23·97
Hairs visible in field of microscope (total for both leaf surfaces)	11·83	6·70	7·25
Leaf index $\frac{\text{length of lamina}}{\text{greatest breadth}}$ of stem leaves (5th node)	1·60	2·41	2·26
Height of plant (cm.)	29	31	33

Comparison with material from south-eastern England showed that all the Shetland plants were hairier. Even those plants which were not referable to the coastal ecotype showed this increased hairiness, although not to the same extent (cf. Turesson's material from the Faroes (see p. 132)). Plants derived from seed of the coastal ecotype from Unst and grown in the experimental garden were hairer than those of the type variety from Surrey. Details are given in another publication (Baker, 1946), in which it is shown that in conditions of exposure plants with hairier leaves tend to be selected and that this would appear to have occurred in the Shetland Isles. Similarly, the average thickness of the leaves of the plants grown from seed was 581 μ (S.D. ± 109), confirming that the greater thickness of the leaves of this type is hereditary. The average leaf-index of the plants grown from seed was 2·48 (S.D. ± 0·51). This result has been commented upon elsewhere (Baker, 1946), for it exemplifies the general tendency towards the selection of individuals with narrower leaves in conditions of greater exposure. Herbarium specimens of the alpine ecotype possessed even narrower leaves.

It was noticed that, at a time during the summer of 1944 when the local plants of *M. dioicum* growing in the Experimental Garden were wilting (although specimens of *M. album* were still turgid), the Shetland plants selected out as being of the coastal ecotype were not. A sample from the top 5 cm. of the soil showed a moisture-content of 3·8% of the dry weight.

In the 1944 season the plants of the coastal ecotype were about 3 weeks later than the local plants in commencing to flower; in subsequent seasons this difference has been narrowed and its significance is doubtful. The growth of plants of this ecotype from seed in another garden at Leeds has shown that they are uniform in passing the first season in a vegetative condition, commencing to flower in the second.

It has been shown above that both ecotypes occur in the Shetland Isles and it is of some importance to gauge their relative frequencies there. A study of herbarium specimens gives the impression that the coastal ecotype is greatly in the majority, but it should be remembered that this ecotype is more striking in appearance than the lowland ecotype and has been taken preferentially by collectors. The lowland ecotype is represented in the herbaria of the British Museum (Natural History), the South London Botanical Institute and the Royal Botanic Gardens (Kew), respectively. Druce (1922) records the lowland ecotype as being quite common in the Shetlands and the coastal ecotype (subsp. *zetlandicum*) as being less frequent. Miss Robina Isbester has confirmed this (in litt.).

Price (1929) observed the coastal ecotype (as *Lychnis dioica* var. *zetlandicum*) growing upon Shetland sea cliffs (see Table 1, p. 133). Miss Isbester informs me that while this ecotype does grow on sea cliffs, in places where it can not be grazed by sheep, it occurs also in places disturbed by cultivation and, in one case, was found on a thatched roof. It is commonly cultivated in gardens and this may be responsible for its subspontaneous occurrence in such situations. Turrill (1928) recorded *Melandrium dioicum* as a weed of cultivated land in Foula. Ostenfeld & Gröntved (1934), after giving a description of a campion which appears to correspond with the coastal ecotype, record that it occurs on fertile rock ledges and sea-fowl cliffs of the Faeroes but add that the species is common in the low-lying regions and is cultivated in gardens.

In addition to the plant from Foula, which represented *M. d.* subsp. *villosum* var. *albiflorum*, Miss Isbester sent seed from a plant which was typical of the coastal ecotype except for its white petals. The seed was identical in appearance with that typical of the ecotype and produced a mixture of plants, with and without anthocyanin, respectively. Another sample of seed from Foula was from a 'pink-flowered plant' of the coastal ecotype. This seed was identical with the previous sample and gave rise to similar plants. The parent plant must have been comparable with plants of *M. dioicum* subsp. *villosum* var. *albiflorum* which show a partial reversion in petal colour (Baker, 1947*c*). Probably Fa 1 was referable to this category as it possesses pink petals and no vegetative anthocyanin. Pink-petalled plants are to be found in the herbaria, and Druce (1922) mentioned that all shades occur from light pink (forma *expallens* Lange) to dark crimson. Ostenfeld & Gröntved (1934) reported a similar range of colour from the Faeroes. Therefore, it would appear that mutation in the direction of the inhibition of pigment formation is fairly frequent and is often followed by a return to partially coloured petals. How often the records of *M. album* from the Shetlands refer to such specimens is problematical.

Druce (1922) has reviewed and doubted the occurrence of *M. album* in the Shetlands, while mentioning having seen white-petalled *M. dioicum*. Saxby (1903) mentioned the occurrence of *M. album* among plants of *M. dioicum* transplanted from the wild state into a garden at Baltasound. It seems likely that a white-petalled variety of *M. dioicum* is referred to again here. Dr Turrill, who has kindly re-examined the specimen collected by J. Gladstone at Freyers, Foula, which is at present in the herbarium of the Royal Botanic Gardens, Kew, agrees that it is probably an albino of the subsp. *zetlandicum* of *M. dioicum*. It was determined previously as *M. album*. Another albino of this description was collected by A. Jackson at Lochend, Northmaren, and grown at Ollaberry. This is preserved as '*Lychnis dioica* no. 108' in W. H. Beeby's collection of Shetland plants at the South London Botanical Institute.

From the results described in this paper, it is apparent that plants of the coastal ecotype show characters which might be expected to accompany polyploidy. The ground-leaves are much thicker than those of the lowland ecotype, the bracts are much thicker and the leaves and stem much hairier. The very stout stem characteristic of this ecotype is maintained in cultivation and the primary roots often reach a centimetre in diameter. On the average, the seeds of the coastal ecotype are about three times as heavy as those of the lowland ecotype and are larger (see Table 2). The diameter of the corolla is larger, although the calyx teeth are not significantly longer. There is a greater depth of petal colour in many plants. All these features have been noticed with polyploids in other genera, and some of them (increased hairiness and larger floral parts) have been described as typical of artificially prepared tetraploid plants of *Melandrium album* (Westergaard 1940; Warmke & Blakeslee, 1940). Furthermore, D. Löve (1942*a*) found her plant of '*M. crassifolium*' to be tetraploid. For these reasons cytological investigations were made.

The large seeds from Haroldswick, Unst, and all the batches of seeds from Foula, were tested by germination on moist filter-paper followed by fixation of the root-tips two or more days after emergence. As a preliminary, smears were made by Warmke's (1935) method using aceto-carmine after fixation in acetic acid-alcohol mixture. Later, permanent preparations of sectioned material were made, using iron alum-haematoxylin stain after fixation in Belling's modification of Navashin's fluid. In all cases twenty-four chromosomes were found (see Fig. 1), indicating that the seed was from diploid plants (cf. Blackburn, 1924, 1928, etc.). Mature pistillate plants received from these two sources were examined by root-tip smears while staminate plants were examined by smearing pollen-mother cells and by measurement of the diameters of pollen grains. No departure from the diploid condition was found and the pollen grains were of comparable diameter (U 2 average 32·75 μ, S.D. ± 1·25; U 3 average 34·0 μ, S.D. ± 0·75; Fa 3 average 38·25 μ, S.D. ± 1·0) with those of plants from Surrey (average 35·0 μ, S.D. ± 0·05).

The apparent contradiction between the presence of those characters which suggest polyploidy and the actual diploid condition is not so remarkable when it is considered that the great majority of character differences between the coastal and lowland ecotypes are in the same direction as those between *M. album* and the lowland ecotype of *M. dioicum*, although *M. album*, also, is diploid (see also remarks by D. Löve, 1942*b*, pp. 263–4 on *Silene maritima* subsp. *islandica*). Another character in which the coastal ecotype tends towards *Melandrium album* is the leaf-index (cf. Baker, 1945*a*, 1946, 1947*a*), both types having narrower leaves than the lowland ecotype of *M. dioicum*. It seems quite possible that these resemblances are expressions of parallel evolution, for both the coastal ecotype of *M. dioicum* and the species *M. album* are adapted to withstand greater exposure than the lowland ecotype of the former species. The lack of tree-cover, and the strong winds encountered in the Shetlands, must demand a more xerophytic nature than is normally possessed by the lowland ecotype. Another resemblance to *M. album* is in the apparent intolerance of shading shown by some specimens cultivated from the seed from Foula.

Wright (1933) recorded plants of *M. dioicum* (as *Lychnis dioica* Mill. from Lundy Island, in the Bristol Channel) which were hairier than usual and tended to bear flowers in terminal clusters. It is unlikely that these plants represent subsp. *zetlandicum* (= the coastal ecotype), for the accompanying photograph shows them to have rounded leaves typical of the lowland ecotype. Wright, himself, discounted the possibility of their being the former because their flowers are smaller (not larger) than normal. It is probable that they represent the extreme hairy form of the lowland ecotype referred to above.

It is possible that the sub-alpine ecotype may be represented in the herbarium material from the four Scottish mainland stations given in Table 1 (p. 133). However, because of personal uncertainty of the differences between the sub-alpine and coastal ecotypes and remembering D. Löve's (1942 *a*) statement that in Sweden '*M. crassifolium*' (the coastal ecotype) and '*M. dioicum* subsp. *lapponicum*' (part of the sub-alpine ecotype) are identical, a more definite statement of the occurrence of the sub-alpine ecotype in this country cannot be made. In view of the fact that *M. dioicum* occurs, for example, up to an altitude of 1061 m. on Lochnagar, above the forest limit, the sub-alpine ecotype may well be represented in such situations. As yet, no evidence has been found of the occurrence of the alpine ecotype in this country.

III. DISCUSSION OF THE HISTORY AND DISTRIBUTION OF THE ECOTYPES

(1) *Modes of origin of ecotypes*

Turesson insisted (1925, pp. 188–9) that the characterization of different regions by different types is brought about by the 'differentiating and sorting effect of the extreme habitat factors upon the population' and that such selection entails progressive loss of power to differentiate ecotypes as the distance from the original distribution-centre increases. This leaves out of account the mutational factors, whether gene-mutation, chromosome-aberration or autopolyploidy. It is more in line with modern concepts of evolution to believe that these factors play a considerable part in the production of new forms upon which natural selection can act. Furthermore, as most differences between ecotypes are of a quantitative nature and may often depend upon polygenes, it is by no means certain that the selection of new combinations of polygenes, already present in an apparently monomorphic type, might not give rise to new ecotypes without mutation (see Mather, 1943). A re-examination of the natures and occurrences of the ecotypes of *Melandrium dioicum* upon the continent of Europe may throw new light upon this and other points.

(2) *Distribution of the ecotypes*

As Turesson maintained (1925, p. 188), the thin-leaved lowland population of Scandinavia probably represents the northern extension of the lowland population of Central Europe. This ecotype is relatively uniform over a wide area.

Although Simmons (1907) separated the subspecies *lapponicum* from the southern coastal type on the basis of increased hairiness, Turesson noticed that this character was not an invariable one in Lapland, for it was possible to find there individuals which, as regards hairiness, match the southern type. It is shown elsewhere (Baker, 1946) that hairiness is dependent, to a certain extent, upon the environment, and there appears to be an ecocline in this character observable as increased hairiness of plants occurring in more and more exposed habitats. In the lowland ecotype this culminates in the plants which Rouy & Foucaud (1896) have referred to *Melandryum silvestre* Röhl. var. *villosum* Celak. and Compton (1920) has named *Melandryum dioicum* var. *villosum* sub.-var. *villosissimum* and a similar variation within the coastal ecotype would not be unexpected. In this case there may be justification for uniting the coastal and sub-alpine forms into one ecotype.

In addition to Turesson's observations upon Simmons's subspecies *lapponicum*, D. Löve has stated recently that this subspecies and '*M. crassifolium*' are identical and implies that they are tetraploid ($2n=48$). However, the situation is not a simple one in which the coastal and sub-alpine ecotypes are mere polyploids for, in addition to the demonstration above that the Shetland plants are diploid, specimens of a 'sub-alpine variety' collected by J. Clausen at Abisko have been shown by Warmke & Blakeslee (1940) also to be diploid ($2n=24$).

According to Turesson (1925, p. 227; 1931) the alpine ecotype is entirely wanting from the Central European Alps. There is some reason for believing this to be incorrect. According to his statement, the alpine ecotype is, in part, identical with the variety *subacaulis* of Norman, the extreme dwarfs forming this part of the ecotype The description given by Norman (1893), and the herbarium material from Spitzbergen and Greenland which has been studied by the present author, have convinced him that the alpine ecotype is represented at least in the following cases (which would be extremes):

(1) De Candolle (1824) described *Lychnis dioica* var. *pygmaea*, 'in monte Margazolo', as being very small, with sub-imbricate leaves and one or two flowers.

(2) Ascherson & Graebner (1929), in their Flora of Central Europe, described *Melandrium dioicum* var. *pygmaeum* which is low, with one to four flowers upon the stem. The leaves are almost imbricate and the flowers smaller than usual. They recorded that it was uncommon and gave as a synonym '*M. rubrum* g. *pygmaeum* Gürcke in Richter-Gürcke *Pl. Eur.* II, 328 (1903) etc.' They listed this quite separately from the var. *subacaule* from arctic Norway.

(3) Rouy & Foucaud (1896) described *Melandryum silvestre* Roehl. *β pygmaeum* Ser. from Cabriac in the Cévennes and they identified this with De Candolle's plant. It was a plant with short stems, very short internodes and sub-imbricate leaves, bearing one to four very small flowers.

It seems that an alpine ecotype does exist in Central Europe, although rarely. This rarity may be due to environmental or historical factors, or to a combination of both. Turesson (1925, pp. 227–8) rejects the influence of the environmental factors, saying that the difference between the conditions in alpine regions in Central Europe and Scandinavia might be expected to cause some difference in the alpine ecotypes found in each, but could hardly be expected to cause the *absence* of the type from one of them. Whether one accepts this argument or not, the alpine ecotype does appear to occur in both regions.

(3) *Historical factors in distribution*

The historical argument used by Turesson (1925, p. 229) is that:

'It is of great significance, however, that subfossil findings of some of these plants from interglacial time have been made (*Melandrium rubrum* in REID (1911)...). These plants have thus been flourishing upon ice-free tundras of Middle Europe, which remained unglaciated during the last epoch of glaciation. Then again it appears probable that it was just alpine ecotypes of these species that endured life on this tundra, at least at the maximum glaciation. That these types followed the northern border of the retreating ice sheet and not at the same time the southern border into the Alps may be explained by climatical differences in north and south, which must have become established soon after the initial melting of the ice causing an early zonation of the vegetation in the way contemplated by WEBER (1915, p. 45).'

The reference to 'Reid (1911)' is to a contribution by Clement Reid to a joint discussion by certain sections of the British Association at their Portsmouth meeting in that year (published in the *Naturalist*, pp. 373-9, 1911). It was reprinted, also, in the *Report of the British Association* for 1911 (pp. 573-7) and is to be found in the *Journal of Ecology*, vol. 1, pp. 42-6, 1913. There is no mention of inter-glacial remains from the Continent and no reference whatever to *M. dioicum*. However, Reid (1899, p. 95) has reported such remains from West Wittering, Sussex, and E. M. Reid and Chandler (1923) record this species from similar beds at Clacton, Essex. In both cases the accompanying flora was *not* one typical of tundra.

Schroeter (1926) pointed out that fossils from the alpine Dryas-clays include not only the typical 'Dryas-flora' but woodland and marsh plants, as well as *M. dioicum*, so that a conclusion is difficult to draw. There does not appear to be any satisfactory demonstration that it is the alpine and not the lowland ecotype which appears in fossil remains. Even if *M. dioicum* has been found in remains from inter-glacial times (and in England at that), this certainly does not prove that it 'flourished on the ice-free tundras of Middle Europe' during the last *glaciation*.

The post-glacial fossil records of *M. dioicum* from Great Britain are from the Boreal 'Lower Forest' beds of the Scottish Southern Upland peat mosses (Lewis, 1905-11), and the sub-Boreal 'Upper Forest' beds of Teesdale (Lewis, 1907). This would appear to indicate that the lowland ecotype was involved. However, on Turesson's reckoning, it is the alpine ecotype which is the most ancient, and the other ecotypes are derived from it by selection, so that the present-day colonization of any high mountain area south of Scandinavia is not possible. Such a theory is intermediate between two possible extremes: (i) the complete elimination of *M. dioicum* form Central Europe with post-glacial restocking from outside refuges or (ii) survival or the lowland ecotype somewhere in Europe. Neither theory is completely unworthy of examination.

If *M. dioicum* were completely eliminated, from whence could the recolonizing plants have come? Turrill (1929, p. 73) pointed out that 'the idea of the Balkan Peninsula as a land of refuge for pre-glacial Central European types is plausible' and (p. 477) he noted, further, that there is no continuous impassable barrier separating Central Europe from the Balkan Peninsula. *M. dioicum* is found in the Northern Balkans at the present day while Turrill (1929, p. 395) includes *Melandrium nemorale* in his list of plants showing Pannonian connexions. These latter are species of essentially Balkan character which have spread northwards into old Austro-Hungarian territories. It is possible that *M. nemorale* may prove to be an extreme shade-form of *M. dioicum* (see Baker, 1945*a*, 1946, etc.). Also, there is the possibility that *M. dioicum* might have spread northwestwards from unglaciated territory outside Europe.

The alpine ecotype has not been noticed in mountainous regions of Great Britain, and there are few records of possible plants of the sub-alpine ecotype even though these mountains were originally to the north of the retreating ice-sheet. If the thick-leaved ecotype passed northwards over Europe (and Britain) following the retreat of the ice, it is surprising that so few examples of the ecotypes which can withstand exposure have persisted in Great Britain. The species, as it is most commonly known here, is a woodland mesophyte. The highest altitude reached in the British Isles by *M. dioicum* is 1061 m. on Lochnagar, Braemar (Wilson 1929–31), and it may be significant that the uppermost limit of forest in Scotland in sub-Boreal time was over 900 m. (cf. Tansley, 1939, p. 173).

These difficulties may be eliminated by assuming that it was as the lowland ecotype that *M. dioicum* recolonized Central Europe following the retreat of the ice and that the alpine ecotype is derived from it. This still leaves the greater abundance of the alpine ecotype in the Arctic and the Scandinavian mountains to be explained. It is quite possible that differences in environmental conditions may have much to do with this, but there is an additional historical possibility.

The lowland ecotype would have followed the retreat of the ice-sheets *both* northwards and southwards. Only the margins of the populations would be subjected to the very unfavourable conditions which were retreating constantly and being followed by more favourable conditions. This is not a particularly likely source of new forms. It is probable that, during the post-glacial climatic oscillations, perhaps at the time of the 'climatic optimum', the northern limit of the lowland ecotype was farther north than is the case at the present day. Such a fact has been amply demonstrated for several species (e.g. review in Wright, 1937). After this, when conditions became more severe, a mass of the population would have become subject to increasingly severe conditions. Increasing selection-pressure would be brought to bear upon the population and in conditions such as these any locally favourable variations would stand increased chance of being selected, even apart from any possibility of a causative action of the environment upon variation. In such circumstances the alpine ecotype might have appeared in some quantity in Scandinavia. It would then be in a position to spread even farther north until its own particular limits were reached.

In the Alps there is evidence of a similar phenomenon in that the upper forest limit was at one time higher than at present (cf. Wulff, 1943, p. 161). However, the area between the two extremes of the upper limit is much smaller than the corresponding 'horizontal' area in the north and the numbers of plants involved might be correspondingly smaller. The possible area for spreading would be restricted, also, and it would be difficult for one mountain which did not develop the ecotype itself to receive it from another. Consequently, the alpine ecotype might be expected to be rarer here than in the north.

Scandinavian biologists (see summary in Faegri, 1937*b*) have shown that a number of flowering plants survived at least the last glaciation in 'nunataks' (chiefly situated on the coast of Norway). Whilst giving an excellent summary of the literature on the subject, Faegri did not mention *M. dioicum* in this connexion. In another paper (1937*a*), he ascribed the difference between the Scandinavian and Central European partial populations to the retention of a greater power of differentiation in the former and discounted the possibility of a wandering of the original gene-centre (cf. Turesson, 1932). There is the possibility that the alpine ecotype might have survived at least the last glaciation in a refuge in Norway and spread to its present Scandinavian and arctic area subsequently.

This does not invalidate the reasoning that it may have been derived from the lowland ecotype, which could have reached Norway during a previous interglacial period, and it does not affect the theory that it was the lowland ecotype which restocked most of Europe, following the retreat of the ice. It is also possible that the coastal (= sub-alpine?) ecotype might have persisted through the last glaciation in the Shetland Isles, for these were not completely covered by ice (see Turrill, 1928, pp. 849–50).

(4) *Necessity for further investigation*

It appears to the present author that the ecotypes of *Melandrium dioicum* are still in need of more study in this country and on the continent of Europe. In Britain, the presence of the sub-alpine ecotype needs confirmation, and its relation to the coastal ecotype should be elucidated. The absence of the alpine ecotype must be made more certain. The presence of *both* the lowland and coastal ecotypes in the Shetlands needs explanation. In Scandinavia, further investigation may show the sub-alpine ecotype to be more heterogeneous than Turesson found. Experimental attempts at the selection of thick-leaved types from thin-leaved and vice versa might show whether one type can arise from another by 'differentiating and sorting effect of the extreme habitat factors upon the population' (Turesson, 1925), or whether some mutation is necessary.

SUMMARY

1. A summary is given of the pioneer investigations by Turesson into ecologically significant variation within *Melandrium dioicum*. Four distinct ecotypes were described from lowland (woodland), coastal, sub-alpine and alpine regions.

2. A report of Turesson's physiological experiments, in which British material was compared with Faeroese and Swedish material, is followed by original observations upon material of the lowland ecotype from south-eastern England and the Shetland Isles and the coastal ecotype from the latter.

3. Material and seeds received from Foula and Unst have been grown in an experimental garden and the results analysed. They show that in the Shetland Isles both the lowland and coastal ecotypes occur (with colour-variation in both cases). The differences between the lowland and coastal ecotypes are tabulated and illustrated. The former is shown to correspond with *M. dioicum* subsp. *villosum* (Compt.) Baker and the latter with *M. d.* subsp. *zetlandicum* (Compt.) Baker.

4. The reported occurrences of *M. album* in the Shetland Isles are reviewed and it is concluded that in the majority of cases, at least, these refer to albinos of the coastal ecotype of *M. dioicum*. The similarities between the coastal ecotype and *M. album* may be due to parallel evolution in response to exposure.

5. Many of the characters shown by the coastal ecotype are often associated with polyploidy. Despite this, all material from the Shetland Isles examined was found to be diploid ($2n = 24$).

6. The possible modes of origin of new ecotypes within a species are discussed and the probable role of mutations is introduced.

7. Scandinavian work on the distribution of the ecotypes of *M. dioicum* is surveyed and the distinction of the sub-alpine from the coastal ecotype is questioned. It is shown that there is no simple pattern of diploid lowland and tetraploid coastal and sub-alpine ecotypes as all these may exist in the diploid state.

8. Turesson's statement that the alpine ecotype is entirely wanting in the Central European Alps is demonstrated to be incorrect and the historical argument based upon this, that the alpine ecotype is the oldest and that all others are derived from it, is contested. Evidence is presented that it was the lowland ecotype which colonized central and northern Europe in the post-glacial period. This ecotype has been reported from British interglacial remains and may either have been eliminated from Europe during glaciation or have survived in a southern refuge. Possible explanations of the greater abundance of the alpine ecotype in Scandinavian rather than Alpine mountains are advanced.

9. The directions which might be taken by further investigations are suggested.

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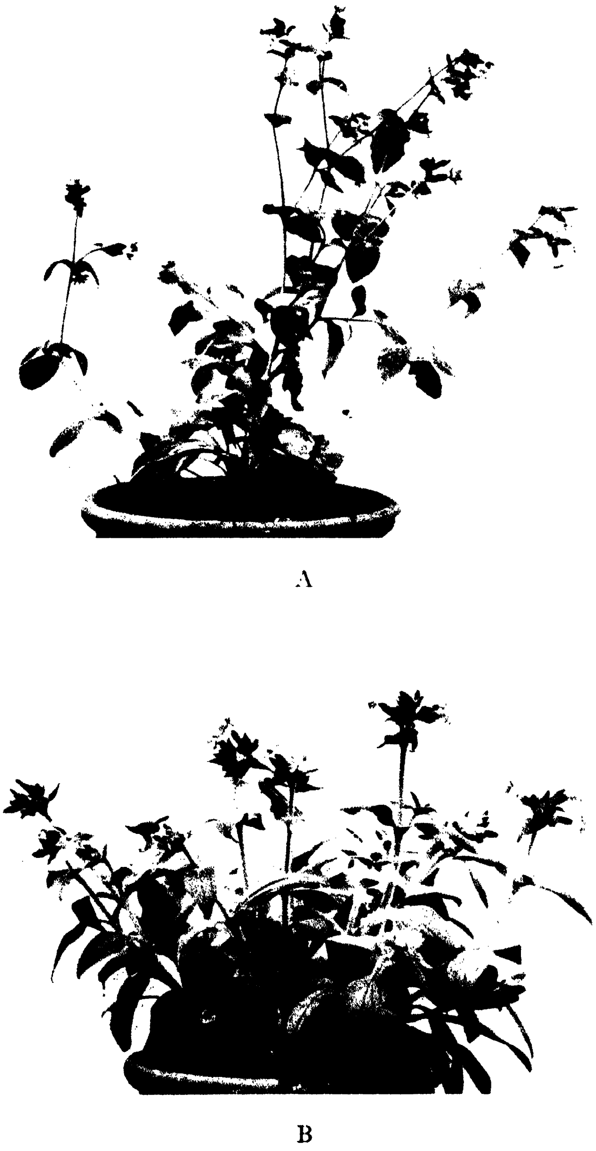
REFERENCES

ÅKERLUND, E. (1933). Ein Fall von Naturselektion in einer Kreuzungspopulation. *Hereditas*, Lund, **18**, 16.
ASCHERSON, P. & GRAEBNER, P. (1929). *Synopsis der Mitteleuropäischen Flora*, vol. **1**. Leipzig.
BAKER, H. G. (1945*a*). The autecology of *Melandrium dioicum* (L. emend.) Simonkai, *M. album* (Mill.) Garcke (*Lychnis*) and their hybrid. Ph.D. thesis, Univ. of London.
BAKER, H. G. (1945*b*). A note on natural hybridization illustrated by reference to *Melandrium dioicum* (L. emend.) Coss. and Germ. and *M. album* (Mill.) Garcke. *Naturalist, Lond.*, p. 129.
BAKER, H. G. (1946). The reaction of plants of the genus *Melandrium* to exposure. *Proc. Leeds Phil. Soc.* (Sci. sect.), **4**, 359.
BAKER, H. G. (1947*a*). Accounts of *Melandrium*, *M. album* and *M. dioicum* for the Biological Flora of the British Isles sponsored by the British Ecological Society. *J. Ecol.* (In the Press.)
BAKER, H. G. (1947*b*). Stages in invasion and replacement demonstrated by species of *Melandrium*. *J. Ecol.* (In the Press.)
BAKER, H. G. (1947*c*). The effects of some insect parasites upon species of *Melandrium*. *Naturalist, Lond.*, p. 13.
BLACKBURN, K. B. (1924). The cytological aspects of the determination of sex in the dioecious forms of *Lychnis*. *Brit. J. Exp. Biol.* **1**, 413.
BLACKBURN, K. B. (1928). Chromosome number in *Silene* and the neighbouring genera. *Z. indukt. Abstamm.- u. VererbLehre*, Suppl. **1**, 439.
COMPTON, R. H. (1920). *Melandryum* in *The Cambridge British Flora* (ed. C. E. Moss), vol. **3**. Cambridge.
DE CANDOLLE, A. P. (1824). *Prodromus Systematis Naturalis Regni Vegetabilis*, vol. **1**. Paris.
DRUCE, G. C. (1922). Flora Zetlandica. Supplement of *Rep. Bot.* (*Soc.*) *Exch. Cl. Manchr.*, 1921, **6** (4), 457.
FAEGRI, K. (1937*a*). Some fundamental problems of taxonomy and phylogenetics. *Bot. Rev.* **3**, 400.
FAEGRI, K. (1937*b*). Some recent publications on phyto-geography in Scandinavia. *Bot. Rev.* **3**, 425.
LEWIS, F. (1905–11). Papers in *Trans. Roy. Soc. Edinb.* vols. **41**, **45**, **46**, **47**.
LÖVE, D. (1942*a*). A tetraploid dioecious *Melandrium* found in nature. *Hereditas, Lund*, **28**, 241.
LÖVE, D. (1942*b*). Some contributions to the cytology of Silenoideae. *Svensk Bot. Tidskr.* **36**, 262.
MATHER, K. (1943). Polygenic inheritance and natural selection. *Biol Rev.* **18**, 32.
NORMAN, J. M. (1893). Flora arcticae Norvegiae species et formae nonullae novae v. minus cognitae plantarum vascularum. *Forh. Vidensk Selsk. Krist.* (Oslo).
OSTENFELD, C. N. & GRÖNTVED, J. (1934). *The Flora of Iceland and the Faeroes*. Copenhagen.
PRICE, W. R. (1929). Notes on the flora of Zetland, 1928. *Rep. Bot.* (*Soc.*) *Exch. Cl. Manchr*, **8**(5), 770.
REID, C. E. (1899). *Origin of the British Flora*. London.
REID, E. M. & CHANDLER, M. E. J. (1923). The fossil flora of Clacton-on-Sea. *Quart. J. Geol. Soc. Lond.*, **79**, 619.
ROUY, G. & FOUCAUD, J. (1896). *Flore de France*, vol. **3**. Asnières and Rochefort.
SAXBY, C. F. N. (1903). *Edmonston's Flora of Shetland*. Edinburgh and London.
SCHROETER, C. (1926). *Das Pflanzenleben der Alpen*. Zurich.
SIMMONS, H. G. (1907). Ueber einige lapplandische Phanerogamen. *Ark. Bot.* **16**, 1.
TANSLEY, A. G. (1939). *The British Islands and their Vegetation*. Cambridge.
TURESSON, G. (1922). The genotypical response of the plant species to the habitat. *Hereditas, Lund*, **3**, 211.
TURESSON, G. (1925). The plant species in relation to habitat and climate. Contributions to the knowledge of genecological units. *Hereditas, Lund*, **6**, 147.
TURESSON, G. (1927). Untersuchungen über Grenzplasmolyse- und Saugkraftwerte in verschiedenen Ökotypen derselben Art. *Jb. wiss. Bot.* **66**, 723.



BAKER—THE ECOTYPES OF *MELANDRIUM DIOICUM*

TURESSON, G. (1928). Erbliche Transpirationsdifferenzen zwischen Okotypen derselben Pflanzenart. *Hereditas, Lund*, **11**, 193.

TURESSON, G. (1931). The geographical distribution of the alpine ecotype of some Eurasiatic plants. *Hereditas, Lund*, **15**, 329.

TURESSON, G. (1932). Die Genenzentrumtheorie und das Entwicklungszentrum der Pflanzenart. *K. fysiogr. Sällsk. Lund Förh.* **2**, 76.

TURRILL, W. B. (1928). The flora of Foula. *Rep. Bot. (Soc.) Exch. Cl. Manchr*, **8**, 838.

TURRILL, W. B. (1929). *The Plant Life of the Balkan Peninsula.* Oxford.

WARMKE, H. E. (1935). A permanent root tip smear method. *Stain Tech.* **10**, 101.

WARMKE, H. E. & BLAKESLEE, A. F. (1940). The establishment of a 4*n* dioecious race in *Melandrium. Amer. J. Bot.* **27**, 751.

WEBER, C. A. (1915). Die Mammutflora von Borna. *Abh. naturw. Ver. Bremen*, vol. **23**.

WESTERGAARD, M. (1940). Studies on cytology and sex determination in polyploid forms of *Melandrium album. Dansk. bot. Ark.* **10**, 1.

WILSON, A. (1929–31). Altitudinal range of British plants. Supplement to *Northw. Nat.* **6** and **7**.

WRIGHT, F. R. E. (1933). Contribution to the flora of Lundy Island. *J. Bot., Lond.*, **71**, Suppl. **6**, 1.

WRIGHT, W. B. (1937). *The Quaternary Ice Age*, 2nd. ed. London.

WULFF, E. C. (1943). *An Introduction to Historical Plant Geography.* Waltham.

EXPLANATION OF PLATE

Plate 3. A. Potted plant of *Melandrium dioicum* subsp. *villosum* (lowland ecotype). Grown in the Experimental Garden, Leeds, from seed from Meanwood, near Leeds, Yorkshire. Seed sown February 1946, photographed July 1947.

B. Potted plant of *M. dioicum* subsp. *zetlandicum* (coastal ecotype). Grown alongside previous specimen from seed from Foula, Shetland Isles.

REVIEWS

The genus Crepis, Parts 1 and 2. By ERNEST BROWN BABCOCK. University of California Press, Berkeley and Los Angeles, 1947. Paper covers $13.50, cloth $15.00. 10¼ × 6⅞ in., pp. 1030, 36 plates, 305 figures.

This extremely full account of a genus of Compositae is dedicated as a memorial to Harvey Monroe Hall and summarizes investigations commenced over 30 years ago. The work is in two parts, with continuous pagination, forming volume 21 and volume 22 respectively of the University of California Publications in Botany. Part One (197 pages) deals with the history of the investigations, the taxonomic concepts, criteria of classification and phylogeny, and special problems of *Crepis* such as phylogeny, geographical distribution, migration, endemism, origin and evolution. Part Two (pp. 199–1030) is devoted to the systematic treatment of the genus.

In the second part, 196 species are recognized, some with subspecies and other paramorphs. Very adequate descriptions, illustrations as black and white text-figures, ranges (by quotation of herbarium specimens studied by the author), and discussions are provided. Ranges or distributions of the species grouped in sections are given in outline maps. Thirty-five plates of photographs of herbarium specimens are reproduced near the end of the part. An index to *exsiccatae* cited, and to generic and specific names completes the work. In other words, we have here a model for taxonomic monographs along modern lines.

In Part One, as already indicated, a large number of subjects of very general interest, are discussed with special reference to problems of *Crepis*. The author makes the very definite statement that 'the systematic treatment of *Crèpis* presented in this monograph rests primarily on comparative morphology'. It is extremely satisfactory to have on record such an accurate and frank break with the modern tendency of claiming a classification as based on phylogeny. With some few exceptions, phylogenic theory is based on classification, not classification on phylogeny. This does not deny the tremendous importance of ecology, genetics, cytology, etc. in providing data for taxonomy, nor, on the other hand, the possible validity of phylogenetic schemes or their interest and use. Very great use is made, in a well-balanced rational manner, of cytological data throughout this present work on *Crepis*. Four trends in karyotype diversity are recognized: a progressive decrease in the basic chromosome number; polyploidy; an increase in asymmetry, leading to chromosomes of unequal length or with one arm longer than the other; and a decrease in chromosome size. A very suggestive remark is made regarding genetical experiments: 'Negative evidence of crossability may be of slight significance, but positive evidence in the form of hybrids, together with data on their fertility, is a valuable criterion of relationship.' The evidence indicates that, within the genus *Crepis*, intra-specific differentiation leads eventually to the origin of new species, given some kind of isolation. The author rejects, for *Crepis*, 'the classification of sexual, homoploid populations as species or subspecies primarily, or crucially on the basis of the degree of intersterility or inter-fertility demonstrated by experimental crosses'.

The criteria of classification and phylogeny are considered in detail under such headings as habit, morphology (of the vegetative and reproductive parts), cytology, and genetics (crossability, vigour, and fertility).

The relationships of the genus are outlined and those of the sections are considered much more fully. A review of the geographic distribution of *Crepis* species and of the genera closest to *Crepis* leads Prof. Babcock to the conclusion that the centre of origin and early development of the genus was in Central Asia, probably in the Tien-Shan-Altai region. The migrations from such a centre to the present range of the genus are correlated with Matthew's hypothetical continental outlines and not with Wegener's hypothesis of continental drift. Four important trends of plant migration in Asia during the Tertiary were important for *Crepis*: a north-easterly, a south-easterly, a south-westerly, and a westerly.

A study of the endemic species of *Crepis* gives results completely at variance with the age-and-area concept of Willis and the concept of 'senescence' fails to explain the existence of narrow endemics, since this group includes not only some of the most primitive but also some of the most advanced species of the genus.

Progressive reduction of chromosome numbers is considered to have been of fundamental importance in the evolution of the genus. Each successive step in reduction from 6 to 3 must have occurred independently in several different species. At present the 4-paired species greatly predominate. Reduction of chromosome numbers involves reduction in linkage groups and this, in turn, may be advantageous in suppressing extreme variants without decreasing the potential variability. However, gene mutation must be of equal or even greater importance in speciation, at least within groups having the same chromosome number. Speciation through gene mutation involves isolation between the differentiating populations. Such isolation may be spatial separation or the consequence of internal mechanism. Spatial separation may be geographic or ecologic and the internal mechanism may be a matter of chromosomal transformation or a result following accumulation of mutated genes. Even single genes may act directly in isolating species genetically. Adaptation, through the natural selection of gene mutations, to gradually increasing xeric conditions has been of special importance in the evolution of *Crepis*.

Enough has been said to justify the remarks made earlier in this review, as to the great value of Prof. Babcock's publication not only to specialists but to all interested in evolutionary and taxonomic studies. It is only by accurate and intensive work similar to that recorded in *The Genus Crepis* that marked progress can be made from what has been called 'alpha' towards 'omega' taxonomy—towards an ideal classification which summates all that can be known regarding the structure and behaviour of the plants classified and which serves a maximum number of purposes in the best possible manner.

W. B. TURRILL

The Nature and Prevention of the Cereal Rusts as exemplified in the Leaf Rust of Wheat. By K. STARR CHESTER, Ph.D. 10½ × 7 in. Pp. xvi + 270, with 19 tables, 11 text-figures and other illustrations. Waltham, Mass.: Chronica Botanica Co.; London, W.C. 2: Wm. Dawson and Sons Ltd. 1946. $5.00.

This work constitutes vol. IV of the *Annales cryptogamici et phytopathologici* inaugurated by S. D. Garrett's *Root Disease Fungi* (1944), with which it is uniform in general appearance, though of larger page size. The author, in his Foreword, states that it is intended '...to serve as a useful aid to those dedicating their investigations to future efforts at solution of the rust problem'. For various reasons, the subject matter is organized about the disease of wheat caused by *Puccinia triticina* Erikss. which is here accorded its first detailed, monographic treatment.

There are fifteen chapters, of which the first comprises a short introductory and historical survey. Here, and throughout the book, the author convincingly portrays the disease as of 'surpassing economic importance', and of no less interest scientifically than the more spectacular visitations of *P. graminis*. The economic aspect receives further attention in chapter II, which includes also an account of methods of estimating the intensity of the disease and of forecasting crop losses. Chapter III deals with the physiological effects of the disease, of which the most interesting appears to be the excessive accumulation of proteins and other forms of nitrogen by the rusted foliage.

Chapters IV, V and VI, headed suscepts, symptomatology, and etiology respectively, provide a detailed account of the fungus and its life history. As defined by the concept of *P. triticina* Erikss., the rust is in practice highly specialized to wheat, with *Isopyrum* as the only functional aecidial host—the balance of evidence being against the *natural* occurrence of aecidia on *Thalictrum*. However, in chapter IV, one would like further information about those other species of graminicolous rusts to which *Thalictrum* is naturally susceptible in the aecidial stage; and it would appear also that the occasional occurrence of *Puccinia triticina* on *Agropyrum* spp. needs to be taken into account relative to the American species *Isopyrum biternatum*, the aecidial host of *Puccinia rubigo-vera agropyrina*.

Considerations of this sort lead inevitably to the question of physiologic specialization, which is very fully dealt with in chapters VII and VIII; to which should be added the discussion of the nature of resistance and susceptibility, forming the greater part of chapter XIV. Here, in the reviewer's opinion, is the best part of the book. The author develops the concept of the *race group*, defined as including all those races the reactions of which may, under certain environmental conditions, duplicate those of others within the same group. On this basis, and by eliminating three of the eight differential varieties hitherto employed, the 129 known physiologic races are reduced to forty-four groups—at least half of which are considered to be either as yet unrecognized duplicates of others, or of excessive rarity. Certain generalizations on the geographic distribution of the groups become possible, whereby the problem of the plant breeder is still further simplified. The physiological basis of specialization is conceived in terms of factors for resistance or susceptibility possessed by the five environmentally stable differential varieties, designated for brevity the '**R-S** factors'. If, for each variety, only one such a factor is postulated (i.e. if the same reaction always, *in a given variety*, expresses the same physiological relationship), the race groups of the fungus will themselves become distinguishable in terms of their response to the **R-S** factors, which are viewed as essential elements in their nutrition, of the nature of specific proteins. Thus, the author is led to the conclusion that the restricted host range of the fungus as a whole, and the physiologic specialization of its several races, may both 'trace down to the presence in wheat of a general pattern of protein metabolism favourable for the rust, and, in certain varieties of wheat, of a few specific proteins required by the rust, the number of which is not large'. This assumption, freely admitted as such, should provide a useful basis for future investigations.

Chapters IX, X and XI deal with the environmental and other factors affecting rust survival and development, and with the epidemiological aspects of the disease generally. Many of the earlier sections of this discussion appear unnecessarily lengthy in view of the *ad hoc* nature of the data they attempt to summarize. The author is more successful in his treatment of the annual cycle of the disease in relation to overwintering and dissemination. Briefly, he establishes the prime importance of overwintering rust, even in trace amounts, for the continuance of the disease, whilst disposing of any carry-over effect quantitatively from one year to another: in accordance with the latter view, he develops the thesis that the intensity of the disease is predictable, some 2½ months before harvest, from the temperature and precipitation during a 'critical month' (itself climatically determined) early in the growing season. A notable feature of the discussion is the insistence that generalizations concerning *P. graminis* are not necessarily transferable to *P. triticina*.

Chapter XII deals with natural, regulatory, and cultural control methods, whilst the following chapter, on fungicidal control, leads to the unexpected conclusion that 'economical and profitable sulphur dusting for leaf rust control on a commercial basis is not beyond expectation'. Control by the use of resistant varieties, however, is obviously preferable; and the book ends with a useful survey of progress in that direction, coupled with the warning that our present resistant commercial varieties are far from immune to all rust races.

There is a bibliography of about 480 titles, covering the field until at least the end of 1944: a valuable feature of the book is the full discussion of recent Russian work, as may be judged from the inclusion of nearly ninety references to papers in that language, nearly all of them consulted in the original. The indexing is good. Very few errors have been detected: two of the dates in the concluding paragraphs of p. 5 are incorrect, and other misprints occur on pp. 4, 95, 140 and 169. Finally, those who are accustomed to the usage of the *List of Common British Plant Diseases* may prefer to see the name 'brown rust' accorded more authority on p. 2, or the scientific name of the pathogen introduced somewhat sooner.

This is an important book, and a worthy successor to the author's stimulating earlier volume, *The Nature and Prevention of Plant Disease* (1942). It fulfils admirably the rather limited purpose indicated for it in the Foreword. But it deserves to be read by many others than the professional plant pathologist commencing the study of rusts. It is on this ground alone, perhaps, that the shortened title of the book—*The Cereal Rusts*—is defensible. (Are the publishers responsible for this?). A volume of this length on such a subject could only demand serious consideration as an essay, or elementary text; whereas this is a monograph, and deserves recognition as such.

T. E. T. BOND

Den Skogliga Rasforskningen och Praktiken. By BERTIL LINDQUIST. Pp. 176, 67 text-figures and photographs. Stockholm: Svenska Skogsvårdsföreningens Förlag. 1946.

In this recently published book, Hr. Lindquist attempts to give a general picture of the problem of genetic deterioration in Swedish forests, and some account of immediate practical and of experimental work undertaken by the various bodies interested in forestry, both from the purely commercial and the more theoretical standpoint, designed to improve the genetic stock. It should be stated at the outset that the book is neither a text-book of genetic silviculture nor merely a popular work designed to have a wider appeal; and it would seem a valid criticism that the style and contents to some extent fail to satisfy the requirements of either type. Nevertheless, the book contains much of interest for British foresters; there is, for example, good reason to believe that the type of situation described for Swedish pine-forests is true of our native Scottish ones.

The author expresses his indebtedness in the preface to Prof. Olof Eneroth, through whom in 1931 he first began work on these problems. The first chapter is to a large extent historical, with reference to early work on the importance of genetic factors in forest trees, notably by the Danish Prof. Oppermann over 40 years ago, who first showed (for oak and beech) that the characters of type of growth and twig development, and also growth rate, are essentially genetically determined, and the Swiss Buerger, whose experiments over more than 20 years demonstrated this also for pine. The more recent work of, for example, Zederbauer, Syrach Larsen, and (in Sweden) Nilsson-Ehle, Sylven and Johnsson is also mentioned.

Chapter II gives an account of the degeneration of Swedish forests, both coniferous (*Pinus silvestris* and *Picea excelsa*) and deciduous (*Quercus*, *Betula*, *Fagus*, etc.), attributable to the elimination, by selective felling, of the best trees, and the survival and reproduction of trees with undesirable qualities. The importance of the seed-parent in natural regeneration and in afforestation is emphasized, a factor which has in the past been completely ignored, when seed for reafforestation has been collected from the most convenient source, often from trees of stunted and irregular growth possessing no desirable genetic qualities. Immediate measures are suggested in the third chapter to improve the stock of forest trees, and, for example in the case of pine, the necessity of choosing narrow-crowned, fine-twigged types as seed-parents is stressed. To that end a preliminary survey of pine in Sweden has been undertaken, and the results of this survey are presented (p. 35, fig. 12). Some account is given here also of transplant experiments, which suggest that there may be no climatic hindrance, for example, to the use of South Swedish pine for afforestation in the North, and indeed that such transplantation may be of value in providing a quicker-growing stock than the local native type.

A long-term view of the problem is taken in chapter IV. Considerable reference is here made to the work of Holger Jensen at Ramlösa, where nurseries of seed-parents of forest trees are established, and production methods previously applied to fruit-growing are being used. Thus experiments in hastening and increasing seed-production by bark-ringing, root-trimming and particularly by grafting are being undertaken. The importance of grafting lies not only in the possibility of rapid and controlled production of seeds of desirable types, but also in providing a method by which a particular tree of which seed may not readily be available may be cultivated and used as a seed-parent (cf. fig. 28).

In the fifth chapter a rather detailed account is given of the desirable commercial qualities of each of the main forest trees, and an explanation given of the system of selecting 'élitstammar'—i.e. individual trees with the desired qualities to be seed-parents. In pine, over 3000 such trees have been registered in Sweden, and yield regular seed for the cultivation of 'élite' material. The assumption that a good phenotype usually possesses desirable genetic qualities is well justified by such selection; and Lindquist further points out (p. 96) that environmental variation markedly affecting a *single* tree in a forest would, from what is known of tree root-systems, be highly improbable, so that a clearly superior individual in close stand in all probability possesses a highly desirable genetic constitution. In the case of birch, the desirable commercial qualities are rather complex, and the genetic selection problems correspondingly greater; Lindquist here emphasizes in addition the value of cytological studies on selected 'élite' trees in view of the prevalence of infertile hybrids between *Betula verrucosa* ($2n=28$) and *B. pubescens* ($2n=56$). The former species is generally speaking of greater value to foresters. Work is in progress with artificially induced polyploids of *Betula* which promises well. Similar importance is attached to Nilsson-Ehle's discovery of 'giant' polyploid *Populus tremula* and his subsequent investigations. The chapter concludes with a discussion of the value of preserving areas of native forest as 'genetic reserves' for the future, in addition to the suggested widespread reafforestation with 'élite' material. The actual use of such material in afforestation schemes is discussed in chapter VI; evidence is here adduced that over large areas of Sweden there is, for example, degenerate spruce and birch forest where formerly good pine grew, and it is stressed that afforestation schemes should be particularly concerned to find the right species for the particular locality.

The last three chapters deal chiefly with the problems, 'educational' and economic, of spreading the genetic improvement technique and results throughout Swedish forestry, and are of less general interest than the preceding ones; although certain problems of wider application are touched upon, as, for example, the question as to how far forest 'degeneration' may be due not to genetic factors but to soil exhaustion (p. 151). In conclusion the author expresses his opinion that the time has now come when the various interests and organizations in Sweden involved in this problem of genetic improvement of forest trees must pool their resources and plan together their future policy.

The book is well illustrated by some 60 photographs and several diagrams, all of which bear detailed explanatory captions which make them of considerable interest *per se* and to a large extent independent of the text. The omission of even a short list of references is unfortunate, for experimental work of considerable general interest is frequently quoted in the text, and the research worker in this field will naturally wish to consult the original accounts in more detail.

The extent to which this work is the outcome of previous ecological studies can best be appreciated by reference to the relevant previous works by the author, a list of which is here appended. It is greatly to be hoped that corresponding studies on British woodlands might yield similar practical results for forestry.

List of relevant papers by B. Lindquist

(1931) Den Skandinavians Bokskogens Biologi. *Svenska Skogsvårdsföreningens Tidskrift*, Häfte 3. (E.)

(1932) *Den sydskandinaviska kulturgranskogans reproduktionsförhållanden.* Centraltryckeriet, Stockholm. (E.)

(1932) Om den vildväxande skogsalmensraser och deras utbredning i N.W. Europa. *Acta Phytogeographica Suecica*, IV, Uppsala.

(1935) Studier over skogligt betydelsefulla svenska tallraser. *Norrlands Skogsvårdsförbunds Tidskrift*, Häfte 1. (E.)

(1938) *Den svenska tallen.* Svenska Skogsvårdsföreningens Förlag. Stockholm.

(1938) *Dalby Söderskog.* Svenska Skogsvårdsföreningens Förlag. Stockholm. (G.)

(1939) Virkeskvalitat och rotnettovärde hos smalkronig och bredkronig tall. *Svenska Skogsvårdsföreningens Tidskrift*, Häfte 1. (G.)

(E.) English summary; (G.) German summary.

S. M. WALTERS

Die Goldhaferwiese (Trisetetum flavescentis) der Schweiz. Eine soziologisch-ökologische Studie. By FRANZ MARSCHALL. Pflanzengeographische Kommission der Schweizerischen Naturforschenden Gesellschaft. Beiträge zur geobotanischen Landesaufnahme der Schweiz, Heft 26. 9 × 6 in., pp. 168, 11 text-figs. Hans Huber, Bern. 1947. 12.50 francs.

Over a quarter of a century ago Dr Braun-Blanquet, writing on 'Prinzipien einer Systematik der Pflanzengesellschaften', urged the study of single types of plant community as opposed to treatment on a regional basis with various assorted communities included. The present work follows this precept, using the standard Zürich-Montpellier concepts, including those expounded by Braun-Blanquet for 'total estimate' of abundance plus cover (Gesamtschätzung), sociability and constancy.

The plant association concerned, styled 'golden-oat grassland', is accordingly defined on the basis of characteristic species. Of these there are six (named on p. 119), and of the six only *Trisetum flavescens* has high constancy as shown in the general table of flora (Table 1; the explanation of the 1–10 scale is embedded in the text, p. 48). *Heracleum sphondylium* would qualify as a second characteristic species of high general constancy, but the author states that its subspecies *montanum* is really the characteristic plant, and this appears in only one locality.

Trisetum is by no means always the most abundant member of the community. Thus one of the areas (No. 64) in the Weisstannental is dominated by *Festuca rubra* together with *Anthoxanthum odoratum*, *Cynosurus cristatus*, *Trifolium repens*, *T. pratense*, *Taraxacum officinale*, *Ranunculus steveni* and *Geranium silvaticum*, of which assemblage none is characteristic of the association and only one is characteristic of the next higher vegetation unit—the alliance (Verband) called Triseto-Polygonion bistortae. *Trisetum* itself is present on this area, but among the forty-one vascular plants recorded there is, besides *Trisetum*, only one other which is characteristic of the association, and both of these characteristic species receive lower marks for total estimate than any of the plebeian group named above. This is an extreme example. In isolation it cannot be quoted as a decisive objection to the christening of such a grassland by the name 'Trisetetum flavescentis', but it does serve to demonstrate, to English-speaking ecologists who are not sufficiently familiar with the Zürich-Montpellier system, what the association-name may mean. This aspect of the system is vigorously defended by the author (pp. 6–7).

Terminology apart, the aggregate of areas studied yields quite a large proportion of species of high or fairly high constancy, so that the association as recognized does possess a very substantial floristic nucleus. In all work of this kind, moreover, reliance must be placed, failing evidence to the contrary, on the field experience of the investigator, and on his consequent ability to recognize his special community. Marschall should qualify handsomely in this way, for he has examined 123 examples of 'Goldhaferwiese' in groups widely spaced in Switzerland.

At the beginning of the monograph he reviews, at some length, other work cognate to his own. This includes little from France and virtually none from Britain. Certainly these sources are not rich in immediately relevant data, although they might have yielded something useful about particular species under varying conditions. The discussion based on less remote literature seems, in part, rather abstract. There is a good deal of concern with the classification of supposedly related communities, and this touches what is, to the uninitiated, the most ethereal aspect of Continental plant sociology.

The association occurs, always under marked agricultural influence (manuring and mowing), at rather high elevations throughout central Europe, with geographical extremes, so far as known, in the Pyrenees and the Carpathians. Details are supplied about soil factors (concisely summarized on p. 33), and climatic and other conditions. In relating these to the vegetation, full weight is given to the powerful influence of management. The usual plant-sociological statistics as well as important individual species are amply discussed. The thoroughness of the work can be indicated by taking an example from the edaphic data. The soils frequented by this 'Trisetetum' in Switzerland are generally fairly light; they are usually podsolized brown earths, exceptionally rendzinas. Of the 214 soil samples tested for pH, 89% were within the range 5·3–6·7; the general occurrence of soils within this pH range, which the author rightly quotes for comparison, has been found as 22% of over 2000 samples of arable and grassland soils in eastern Switzerland. This latter source of data included 50% of alkaline samples.

In this connexion one is tempted to compare the occurrence of *Trisetum flavescens* in Britain, despite our lack of correspondingly copious data centred on this particular grass. As an appreciable component of grassland, the species is known on weakly acid British soils, but its most regular occurrence is undoubtedly on chalk and limestones. This contrast in behaviour, as between more southerly and more northerly European regions, repeats a phenomenon known in other species, e.g. *Bromus erectus*. For the grassland type rather than its species, such a comparison cannot be pursued in detail, if only because of the problem of what vegetation to compare; for Marschall's 'Trisetetum flavescentis', as defined by particular characteristic species, does not occur in Britain, and there is probably no true homologue.

The British reader may lose his bearings in this paper, which, like others from Switzerland and Germany, has inconspicuous sub-headings. The good printing, however, and careful composition, make it difficult for the reviewer to complete his task by finding minor errors. There is one in the table of contents, where (p.) 89 should read 87; and the table on p. 89 contains two figures in unexplained heavy type which disrupt the convincing presentation of constancy of certain species in relation to direction of slope. These trivialities do not affect the substantial contribution made by this memoir to the flow of ecological knowledge which we have learnt to expect from Switzerland.

J. F. HOPE-SIMPSON

North American Species of Mycena. By ALEXANDER H. SMITH. 9 × 6 in. Pp. 521, with 56 figures in the text and 99 plates. Michigan University Press; London: Geoffrey Cumberlege. 4 December 1947. Price ($ 6.00) 32*s*. 6*d*.

The limits of the genus *Mycena* were defined with considerable precision by Robert Kühner in his monograph on the genus in 1938. Dr Alexander Smith departs only slightly from Kühner in the placing of these limits. Nevertheless, in splitting the genus into four, as opposed to two, subgenera, and in the relative weight he gives to certain characters, Smith's treatment differs from that of Kühner rather sharply. Owing partly to its technical difficulties and to the need for dried spores and flesh for obtaining a positive amyloid staining reaction with iodine, this test is not used as a primary character in generic subdivision. Smith, indeed, doubts if it is of outstanding theoretical importance in this genus at all. His four subgenera are separated mainly on morphological grounds and are of very different size. *Eumycena*, the largest, contains some 185 American species whilst the others contain twenty-one or fewer. Morphological criteria are again employed to define groups of species within the larger subgenera, and, on the whole, use is made of microscopic and microchemical characters at the lower levels only. The aim is to achieve a convenient and workable system within the generic limits and to place species having numerous features in common in close juxtaposition.

Some 218 species of North American Mycenae are described in detail, and about half of these are illustrated in plates by excellent life-size photographs. These plates give a very good representation of habit, range of form, size and, in most species, a view of gill arrangement. Text-figures are employed to show detail of spore and cystidial form, and although somewhat crowded, they are clear and are arranged to allow easy comparison of related species.

The descriptions of the species in the text are based, in the main, on the author's own observation and include very valuable notes stressing particular points of difference from, or resemblance to, related species, in structure or in habitat. Where the author has insufficient knowledge of any species in the live state, he quotes descriptions from other authors with suitable notes. The criticism of these diagnoses is one of presentation. The points relating to different organs, pileus, stipe, gills, etc., might well have been given separate paragraphs to facilitate quick reference.

The book is complementary to that of Kühner in one important respect. Many of the American species were known to that author only in the form of dried material, often, indeed, sent to him by Dr Smith himself. Now we are presented with detailed descriptions of many of these species based upon careful observation and collection of living and preserved material from many sources. Smith has not only seen a great deal of the preserved material from North American herbaria but also gives a list of his sources, with catalogue numbers, in the descriptions of each species.

The species collected from the North American tropics and subtropics and described by Murrill are not included in the general text, but are given a separate treatment in a section of some seventeen pages. They are keyed as far as possible by means of such microscopic characters as are visible in herbarium material. This treatment is in keeping with the author's principle to rely as far as possible on his own knowledge of species in the living state or, failing that, upon descriptions which include sufficient macroscopic and microscopic detail to allow a reasonable assessment of their affinities.

The binding is strong and serviceable and in these days of austerity in this country it is a pleasure to handle such a book. Its size and weight preclude its use in the field but that is not its purpose and they do not diminish its value to British mycologists. As a work of reference giving figures, and perhaps photographs, as well as detailed and original descriptions of *Mycena* species, it is excellent, and the introductory chapters will be valuable for information and for methods to those who wish to obtain an appreciation of the genus *Mycena*. The inclusion of an index to the new species, new names and new combinations, and a detailed comparison of the limits of *Mycena* as described by Kühner and by Smith would have been very useful. In spite of such relatively minor omissions it will be found a very valuable book.

J. L. HARLEY

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STUDIES IN THE MORPHOGENESIS OF LEAVES

I. AN ESSAY ON LEAF SHAPE

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(With 13 figures in the text)

CONTENTS

	PAGE
Introduction	153
Heteroblastic development	154
Analysis of heteroblastic development	156
(1) The quantitative description of leaf shape	156
(2) Leaf shape and cell size	158
(3) The relation of leaf shape to external and internal environment	160
(4) The genetical control of leaf shape	166
Heteroblastic development and physiological age	168
Conclusion	173
Summary	173
References	174

INTRODUCTION

Studies in the morphogenesis of leaves are in progress in the University of Manchester, and the results will be published in subsequent contributions to this *Journal*. The purpose of these studies is to throw light on the processes which determine leaf shape, and in particular on the processes which determine changes in shape from leaf to leaf on the same plant.

Many researches in morphology, physiology, and genetics converge on the subject of these studies. These researches have not hitherto been brought together and reviewed in the context of contemporary botany. It is appropriate, therefore, to begin this series of papers with an essay on leaf shape, which defines the problems being studied and assembles some published information and some original investigations relevant to the subject.

Botanists are familiar with the fact that in many plants successive leaves on the stem are not all alike. Lower leaves may differ in shape completely from upper leaves, as in *Campanula rotundifolia*; or there may be a gradual transition from one shape to another, as in some varieties of *Gossypium*. The transition in leaf shape may occur as changes in the number of leaflets (e.g. in *Thalictrum flavum*); or in the amount of dissection of the leaves (e.g. in *Daucus carota*); or in the production of an auricled base (e.g. in *Rumex acetosella*); or in the ratio of length to breadth (e.g. in *Polygonum cuspidatum*). On some plants the change in leaf shape is clearly due to external influences: this is so for the aerial and submerged forms of *Ranunculus aquatilis*; on other plants (e.g. *Hedera helix*) there is no obvious external cause for the change in leaf shape from top to bottom of the stem.

HETEROBLASTIC DEVELOPMENT

Some forty to fifty years ago many observations were recorded of the so-called youth forms of leaves and other types of heterophylly. Goebel (1898) put forward the view that a growing plant passes through a course of development, culminating in the formation of reproductive organs, and that this course may be accompanied by changes in leaf morphology from node to node. Where the youth and adult forms of leaves are strikingly different, Goebel referred to their development as heteroblastic; where there are only slight differences between youth and adult forms of leaves, Goebel referred to their development as homoblastic. Examples of heteroblastic development are given in Fig. 1.

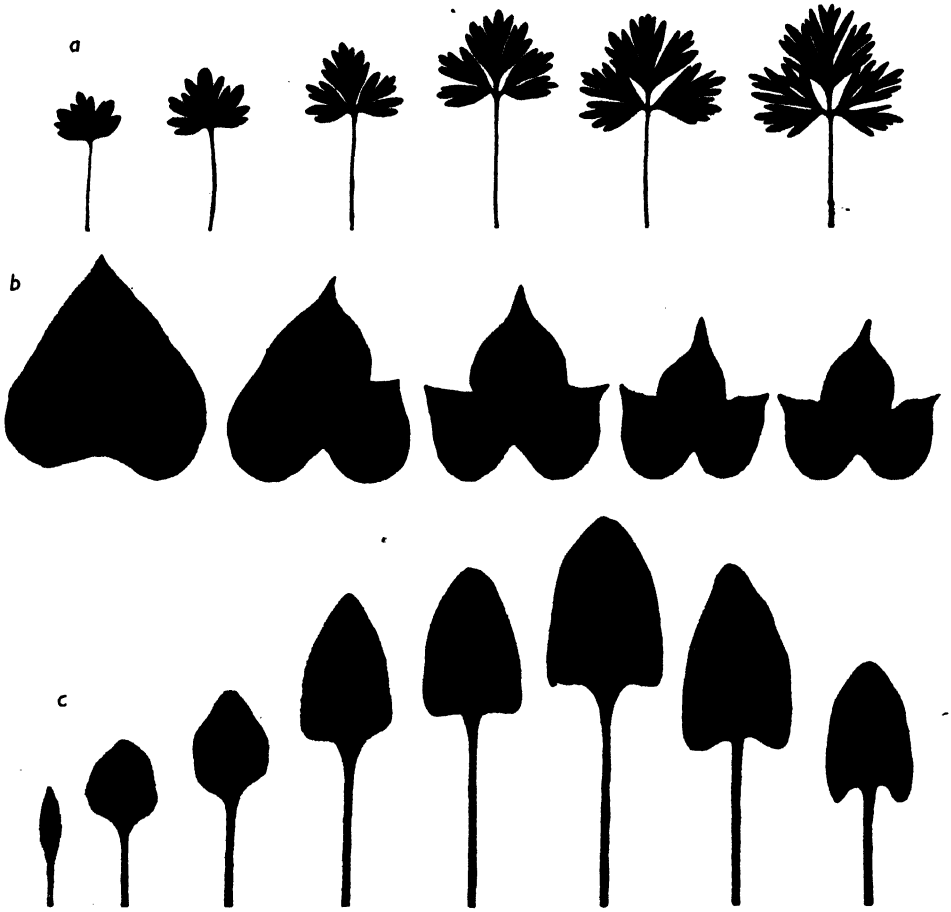


Fig. 1. Examples of heteroblastic development: (*a*) first six leaves of *Delphinium ajacis* (original); (*b*) first five leaves of *Ipomoea caerulea* (original); (*c*) first eight leaves of sugar-beet (data from Belskaya, 1940). For further explanation, see text.

At that time the chief interest in leaf shape lay in its possible significance to phylogeny. The paramount question was—Does the ontogeny of heteroblastic development in a species recapitulate the phylogeny of leaf shape in its ancestral forms? Bitter (1897) examined the comparative morphology of the leaves of Ranunculaceae and Umbelliferae with this end in view. Jackson, having studied reversions to the youth form among leaves of several plants, published in 1899 a paper in which he put forward the view that 'throughout the life of the individual, stages may be found in localized parts, which are similar to stages found in the young, and the equivalents of which are to be sought in ancestral groups'. In seeking examples of Jackson's 'law', other botanists (e.g. Cushman, 1902, 1903, 1904) published observations on heteroblastic development, and discovered it to be very widespread. Among plants showing striking changes in leaf shape from node to node, Cushman described *Chelidonium majus*, *Chenopodium album*, *Potentilla tridentata*, *Arabis albida* and *Chrysanthemum speciosum*. G. H. Shull (1905) published a detailed study of leaf shapes in *Sium cicutaefolium*. He showed that in this plant there is a gradual transition in the first eight leaves of a seedling, from a lobed to a pinnate condition; this is followed by a reduction in size (but not in number) of pinnae toward the end of the growth season. *S. cicutaefolium* is a perennial, and every spring there are differences in shape between the first few leaves produced and the later, summer leaves. The youth form of leaf reappears in 'rejuvenated' buds, so that a shoot growing from the axil of a pinnate leaf, may, nevertheless, produce only lobed leaves. Shull concluded from his work that this variation of leaf shapes was not a case of recapitulation of phylogeny, but was to be explained as the result of physiological processes controlled by the environment. Goebel had come to a similar conclusion from his studies of heterophylly in water plants. By 1905 the recapitulation theory of ontogeny was falling out of favour among botanists, and further studies of leaf shape were not pursued along these lines.

In 1906 Diels revived interest in leaf shapes by tracing the relation between leaf shape and sexual maturity (Blütenreife). He studied the transitions in leaf shape from youth to adult forms in a variety of plants from Europe and Australia, in order to discover whether sexual maturity corresponds with a definite stage in the succession of leaf shapes. His work yielded no clear-cut result: flowering did not always correspond to a definite stage of vegetative development as determined by leaf shape. However, Diels was able to conclude that the external conditions which hasten flowering also hasten the transition from juvenile to adult leaf shape. The leaf shape at any stage was, in Diels's opinion, a summary of the previous environments under which the plant had grown.

In his monograph on experimental morphology, published in 1908, Goebel put forward the hypothesis that youth forms of leaves are a response to poor nutritive conditions, and he cited a considerable amount of experimental evidence to support this view, in particular experiments in which he produced youth forms of leaves on adult plants by cultivating them in warm, moist, shady conditions. Goebel's simple hypothesis still holds the field, though it has not stood unchallenged. In two papers which are discussed later in this essay, Lundegårdh (1914) and Kranz (1930) described experiments which are inconsistent with the view that juvenile foliage is merely the result of poor nutrition.

Goebel carried the subject as far as it could be carried, considering the state of plant physiology at that time. The study of leaf shape has recently been revived; its purpose now is not to illuminate phylogeny but to explore the field of causal morphology. The revival has been led by E. W. Sinnott and his students, who have successfully applied techniques from genetics and physiology to the study of shapes in fruits and leaves. The contemporary plant morphologist regards the shape of an organ as the end-product of three determinants: (i) the shape of its primordium; (ii) the number, distribution, and orientation of cell divisions; (iii) the amount and distribution of cell enlargement. Each of these three determinants is influenced by environment and heredity, and these influences can, if sufficient information is available, be expressed in terms of cell chemistry. There is reason to expect that with this approach to the problems of leaf shape, some of the phenomena described by Goebel and his contemporaries may be more fully understood.

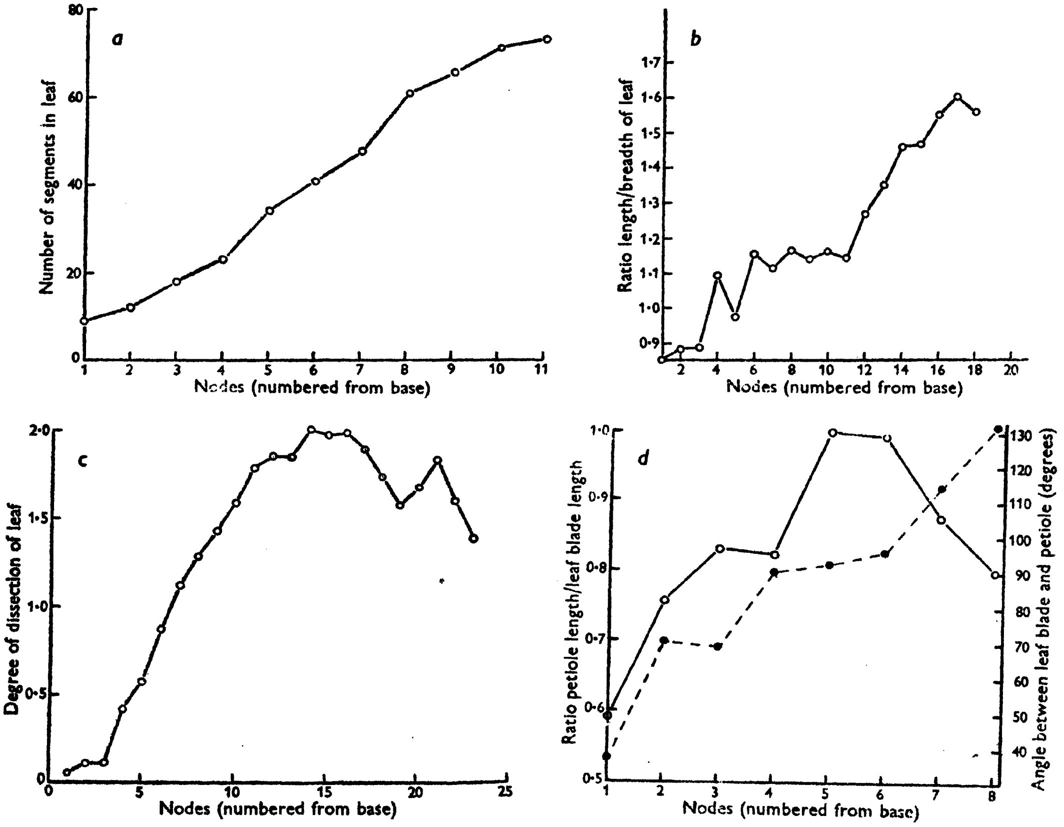


Fig. 2. Graphical representation of heteroblastic development: (*a*) *Delphinium ajacis*, number of leaf segments, plotted against node of insertion; (*b*) *Polygonum cuspidatum*, ratio of length to breadth of leaf blade, plotted against node of insertion; (*c*) *Gossypium hirsutum*, degree of segmentation of leaf blade, plotted against node of insertion; (*d*) sugar-beet: continuous line is the ratio of petiole length to leaf blade length, and broken line is the angle between the base of the leaf blade and the petiole; both plotted against node of insertion. (All original; data for (*d*) from Belskaya (1940).)

ANALYSIS OF HETEROBLASTIC DEVELOPMENT

(1) *The quantitative description of leaf shape*

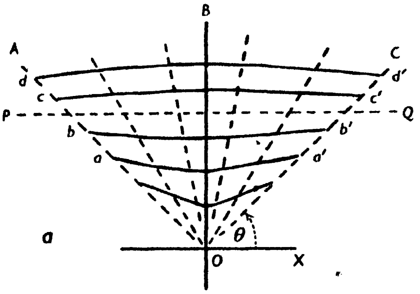
The first step toward an analysis of leaf shape is to express shape quantitatively. There is no general method of doing this: the method to be used depends on the leaf shape being studied.

A simple example of heteroblastic development is expressed quantitatively in Fig. 2*a*. In this plant, a garden variety of *Delphinium ajacis*, there is an approximately linear relation between the number of segments on a leaf and its node of insertion on the stem. A second example is given in Fig. 2*b*; in this instance (*Polygonum cuspidatum*) shape is measured as the ratio of length to breadth of leaf. A third example is given in Fig. 2*c*; in this instance (*Gossypium hirsutum*) change in shape is measured as the mean depth of segmentation between lobes. This measure of shape produces a curve which reaches a maximum and then falls. A cyclic change, such as this, in the shapes of successive leaves is common; it can be seen also in sugar-beet (Fig. 2*d*), in some varieties of *Ipomoea*, and in the common dandelion.

There is an evident regularity in the change of shape from leaf to leaf in these examples. In seeking the causes which underlie this regularity, one naturally inquires first whether the individual leaf passes through similar changes in shape as it unfolds from the bud. On this point there are some data. For instance, Hammond (1941), using the so-called allometry formula, demonstrated that many leaves change their shape in a regular way during growth. She measured the length and breadth of leaves at different stages of growth, from a variety of plants, and found that for any one species the values fall closely on a straight line, $\log y = k \log x + b$, where y = length of leaf, x = breadth, and k and b are constants. This linear relation means that the ratio of relative growth rates along the length and breadth remains constant during leaf enlargement. If $k = 1$, the leaf enlarges without changing shape. If k is greater or less than 1, the enlargement of the leaf is accompanied by a change in shape. No estimates of error are attached to Hammond's values for k, but it is reasonable to conclude from her data that in some plants (e.g. *Polygonum cuspidatum*) the leaf does not change shape as it enlarges, whereas in other plants (e.g. *Linaria vulgaris*, *Smilax rotundifolia*) the leaf changes its shape considerably, and according to a simple rule, as it enlarges. Analogous data, analysed by the same technique, have been recorded by Pearsall (1927), by Delisle (1938), and by Whaley & Whaley (1942); and Schüepp (1945), who put forward a somewhat artificial scheme, based on allometry, to describe the shape of a pinnate leaf, has applied it to *Aspidium filix-mas* and *Delphinium elatum*.

Beside the use of the allometry formula to summarize the changes in shape of leaves during their enlargement there is another interesting, though academic, approach to the problem of leaf shape: namely to consider the shapes of certain leaves to be based on conchoid or cardioid curves of the general form $r = k \operatorname{cosec} \theta \pm b$ or $r = a(1 - \cos \theta)$, where θ is the angle between a nerve and the polar axis, and r is the polar co-ordinate of the edge of the leaf. Sporn (1926) showed how quite complex leaf shapes might be fitted into such a pattern as this; and Smirnov & Zhelochovtsev (1931) demonstrated the resemblance between the observed shapes of a *Tropaeolum* leaf at different stages of growth and a set of conchoid curves. A fully grown leaf of *T. majus* has a smooth and elliptical outline, whereas the same leaf in the bud has six deep incisions (Fig. 3*b*). If the outlines of each one of these incisions are plotted on polar co-ordinates (Fig. 3*a*) and the changes in outline are drawn on the assumption that each incision is in the middle of a conchoid curve (the lines *aa'*, *bb'*, *cc'*, ... in Fig. 3*a* and *b*), then the curve straightens out on paper somewhat as each segment of the leaf outline straightens out during growth. This is a somewhat Procrustean treatment of the data; nevertheless, it has some biological interest: for it implies that in the zone *AOC*, for instance (Fig. 3*b*), the vectors of growth rate along the radii *OA*, *OB* and *OC* are equal, and the same would be true of the vectors of growth toward lobes and incisions in other zones of the leaf. If this were so, the disappearance of lobes during the enlargement of a *Tropaeolum* leaf would not be due to differential growth along different radii; it could come about by *equal* increments of growth along the radii in each zone containing an incision. There are no adequate data to test this speculation, though the results published by Whaley & Whaley (1942) do not support it.

Clearly there are adequate techniques for expressing change in shape of leaves, and there is general agreement that many leaves change shape during enlargement in such a way that the ratio (k) of their relative growth rates in two dimensions is constant. The allometry formula can be used also to express heteroblastic development quantitatively. Two examples will suffice to illustrate this. Delisle (1938) showed that the first five seedling leaves of *Aster novae-anglicae* have different values of k for the ratio of length to breadth of leaf: therefore their final shapes were different, and the change in k from leaf to leaf is a measure of the amount of heteroblastic development. Stephens (1944) used the allometry formula to analyse for genetical purposes the shape of fully expanded leaves in cotton. He obtained values for the regression coefficient of logarithms of leaf length on logarithms of lobe width for successive fully expanded leaves. Since leaf size increases from the first to the fifteenth node, the value of the regression coefficient measures approximately one parameter of the change in shape from node to node. Stephens's data are discussed below under § (4), p. 167.



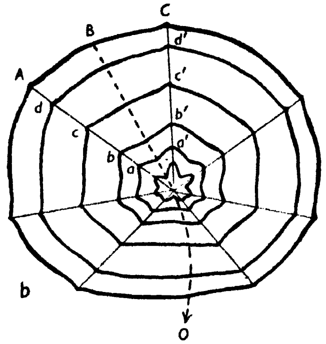


Fig. 3 (*a*). Series of conchoid curves of the form $r = k \operatorname{cosec} \theta \pm b$, where θ is the angle from the polar axis OX and b is the distance above or below the line PQ. The interest of the formula is that each curve aa', bb', cc' ... dd' lies at a constant distance from PQ measured along the lines radiating from O. This implies that a curved front of cells aa', growing at equal rates along OA, OB and OC, would flatten out as it advanced to successive fronts bb', cc'... dd'.

Fig. 3 (*b*). Outline of a *Tropaeolum* leaf at different stages of growth. The successive outlines of a segment AOC approximately correspond to the curves in Fig. 3 (*a*). This suggests that the vectors of growth rate along OA, OB and OC are equal. (Redrawn from Smirnov & Zhelochovtsev, 1931.)

(2) *Leaf shape and cell size*

In the analysis of leaf shape it is essential to know to what extent differences in size and shape are determined by cell divisions, and to what extent by cell expansions. A considerable amount of work has been done on cell size in leaves; this has been summarized by Maximov (1926) and by Tumanov (1932). Of particular relevance to the present essay is Zalenski's work on cell size in successive leaves of *Dactylis glomerata*. Zalenski found (1904), and his results have been confirmed by other workers on other plants, that the size of epidermal cells in successive leaves decreases from node to node up the stem. There is some evidence that this trend of decreasing cell size with increasing height of insertion of leaf may be due to competition for water, which is said to be greater among the upper leaves than among the lower leaves on the stem. It is clear from Zalenski's data (though the matter does not seem to have received any attention) that there is a negative correlation between cell size and leaf size in the first seven leaves of *D. glomerata*, and that a change in shape (measured as the ratio of length to breadth of leaf) accompanies decreasing cell size. The same conclusions emerge from Yapp's work on *Spiraea ulmaria* (1912). Data on *Ipomoea*, to be presented in the second contribution to this series, enable some further analysis to be made of these relationships; it will be shown that the factors which cause smaller cell size do not always cause smaller leaf size too.

As to the possibility of an effect of cell size on leaf shape, it is natural to inquire to what extent changes in leaf shape are due to localized changes in the relative growth rate or division rate of cells in the leaf blade. On this subject there are very few data. The two most significant contributions are from Avery (1933) and von Papen (1935). Avery made a quantitative anatomical study of the development of tobacco leaves. By marking a lattice in ink over a young leaf and observing the way in which the lattice was distorted as the leaf grew, Avery was able to show that the rate of increase in area was not uniform all over the leaf: it was greatest at the base of the leaf around the midrib, and it fell off toward the edges and toward the tip. This is illustrated by Fig. 4 which is a drawing of a model constructed from some of Avery's data. Richards & Kavanagh (1945) carried the analysis of Avery's data further by resolving the rates of increase in area into vectors in two directions at right angles. In this way they were able to express the anisotropic growth rates quantitatively, and it should be possible, by extending their method, to express shape as the result of differential growth rates in different parts of the leaf.

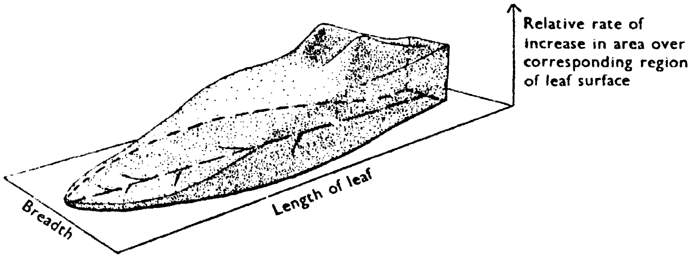

Relative rate of increase in area over corresponding region of leaf surface
Breadth
Length of leaf

Fig. 4. Three-dimensional model to illustrate the relative rate of increase of area at different regions of the surface of a leaf blade. The base of the model is the outline of a tobacco leaf, with its apex to the left. As the leaf expands, the rate of increase in area is not equal all over the leaf surface: increase in area takes place more rapidly at the base of the leaf than at the top. Vertical ordinates, erected on the outline of the leaf blade, form the surface of the model: these ordinates represent the relative rates of increase in area over the corresponding regions of the leaf surface. There are two regions of maximum relative rate of increase of area, toward the base of the leaf on either side of the midrib. (Constructed from data by Avery (1933).)

Von Papen (1935) examined the distribution of size of epidermal cells over the adaxial surfaces of leaves from early stages (e.g. leaf areas of 0·03 mm.^{2}) to maturity. She found in general that in young leaves there was great variation in cell size from place to place on the leaf surface; in mature leaves the variation had almost entirely disappeared, and all cells on the adaxial epidermis were about the same size. Thus, in young leaves of *Tropaeolum*, 0·9 cm. long (Fig. 5), there are distinct indentations, and there are pockets of smaller cells at the base of the indentations. When *Tropaeolum* leaves are about

2·7 cm. long the indentations have almost entirely disappeared, and so have the pockets of smaller cells. These data, taken together with data von Papen collected for other lobed leaves (e.g. *Coleus*, *Saxifraga*) and supported by data from other workers (e.g. Whaley & Whaley, 1942), indicate that changes in shape during the growth of an individual leaf may occur through a 'staggering' of the time of expansion of local pockets of cells in the young leaf.

One instance has been recorded (by Abbé, Randolph & Einset, 1941) where changes in leaf shape from node to node can be attributed to changes in the shapes of successive primordia. These authors observed that in one strain of maize the relative width of the adult leaf increased by about 9% from node to node; and that this increase was due largely to an increase in the circumference of the leaf primordia from node to node. Differences in shape in this strain of maize were due to differences in cell number, not in cell size.

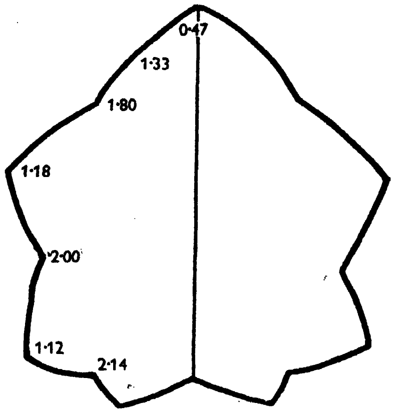


Fig. 5. Outline of leaf of *Tropaeolum*, 9 mm. long. The figures represent the number of adaxial epidermal cells per thousandth of a square mm. Cells on the edge of the leaf at the base of the indentations are smaller than cells on either side of the indentations. These local differences in cell size vanish when the leaf is fully expanded and the indentations have disappeared. (Data calculated from von Papen, 1935.)

(3) *The relation of leaf shape to external and internal environment*

It is common knowledge that the environment may considerably influence the size of leaves. Observations on the effects of environment on the shape of leaves are less familiar; nevertheless, there is a body of data, some of it directly relevant to the theme of the present essay.

Goebel's monograph (1908) on experimental morphology cites experiments in which conditions of low light, together with high temperature and humidity, cause plants to persist in producing juvenile leaves, or to revert to the formation of juvenile leaves after adult leaves have been produced for some time. It was Goebel, too, who demonstrated that the submerged leaves of some aquatics are not formed in response to the water medium *per se*, but to the low light intensity which accompanies submergence in water. Goebel's final conclusion, that most (though not all) forms of heteroblastic development were related to the conditions of nutrition, invited a systematic study of the effects of environment on leaf shape, but apart from a few papers which support but do not extend Goebel's conclusion (summarized by Woltereck, 1928), the systematic study was not at that time undertaken.

Much more recently Åberg (1943) made a thorough study of the effects of light intensity and quality on leaf shape in *Lobelia dortmanna*. He found that the ratio of length to breadth of leaf varied considerably and in a regular way, according to the intensity and wave-length of light falling on the plant. The effect of light intensity on the ratio of length to breadth of adult leaves at the same height of insertion is shown in Fig. 6*a*. The ratio of length to breadth changes regularly from node to node, and increases with decreasing light intensity. These changes in shape are due entirely to changes in cell length in the long axis of the leaf. Åberg suggests that light intensity acts on leaf shape by affecting the carbohydrate balance, which in turn affects the length of the cells in the direction of the long axis.

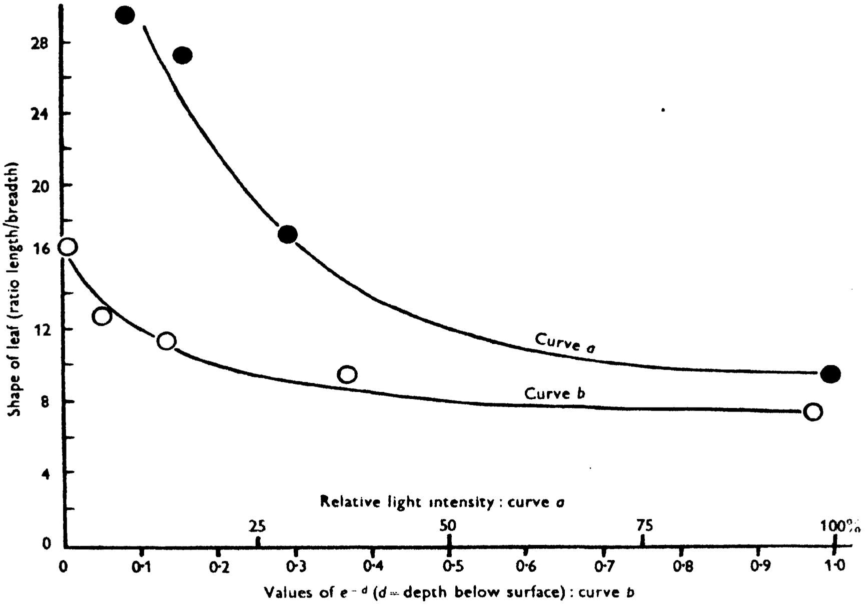


Fig. 6. Leaf shape (length/breadth) of adult leaf of *Lobelia dortmanna* plotted against light intensity (curve *a*), and against e^{-d} (curve *b*), where d = depth of submergence below lake surface. (Calculated from data of Åberg, 1943.) For further explanation, see text.

Åberg, having established that the ratio of length to breadth of leaves increases with decreasing light intensity, proceeded to show from observations of plants in natural habitats, that this ratio increases regularly with increased depth of planting below the water surface; and further that the effect of depth on leaf shape was not due to water *per se*, but to the lower light intensity which accompanies greater depth. Lastly, by cultivating plants of *Lobelia* in shallow troughs, at different depths of submersion, Åberg was able to reproduce data for variation in ratio of length to breadth of leaves, which are strikingly similar to the data he had previously obtained for variation at different intensities of light (Fig. 6, curve *b*).

Data which support Åberg's conclusion have been recorded by Orshansky (1938), who showed that seasonal leaf dimorphism (i.e. the production of 'summer' leaves and 'winter' leaves) in *Ononis natrix* can be attributed to light intensity. By cultivating plants in the shade Orshansky produced 'winter' leaves in summer.

The results of Åberg and Orshansky support Goebel's view that leaf form is influenced by nutrition, especially by the level of carbohydrate nutrition. Further support for this view comes from a paper by Gessner (1940), who claims that he has produced typical aquatic leaves of *Ranunculus baudotii* by growing plants in air, in the absence of carbon dioxide. Gessner's claim needs to be supported by further experiments; if it were established, it would indicate a very close causal connexion between carbohydrate metabolism and heterophylly.

There is some evidence that mineral nutrition affects leaf shape. Pearsall & Hanby (1925) proved that the ratio of length to breadth in *Potamogeton perfoliatus* depends upon calcium and potassium levels. In excess calcium the ratio of length to breadth is $1{\cdot}72 \pm 0{\cdot}18$; in excess potassium the ratio is as high as $3{\cdot}38 \pm 0{\cdot}62$. Mothes (1932) demonstrated that the morphology and anatomy of *Nicotiana rustica* depends on the level of nitrogen nutrition relative to the concentration of other salts. Low nitrogen, accompanied by low concentrations of other salts, produced xeromorphic leaves with small cells and high stomatal frequency; low nitrogen, accompanied by high concentrations of other salts, produced succulent leaves with large cells and low stomatal frequency. Similar effects of the salt balance on leaf form are evident from experiments described by Keller & Poplavsky (1934), and by Söding (1934).

Notwithstanding these examples of the influence of carbohydrate and mineral nutrition on leaf shape, there is no strong evidence that changes in the levels of these substances in plants are responsible for heteroblastic development; and there is a good deal of evidence against such a view. A much more promising hypothesis is that some instances of heteroblastic development are due to a response of leaf shape to length of day, so that spring and autumn leaves are similar to each other but unlike summer leaves. Some examples will illustrate this point. Krenke (1940) found a progressive decrease, from node to node, of the angle at the base of the leaf blade of *Morus alba*. The rate of decrease of the angle, from node to node, is more rapid in a 10 hr. day than in a 16 hr. day. In the same year, Harder & von Witsch (1940) showed that the leaf shape of *Kalanchoë blossfeldiana* is affected by length of day. In short days leaves are succulent, with entire edges, and sessile; in long days leaves are thinner, with notched edges, and petiolate. Their experiments lead the authors to suggest that under short days some substance is produced which affects leaf shape, and which can migrate up the stem to affect the leaf shape of leaves in the same orthostichy, above the leaf which produces the substance, causing a short-day type of leaf to develop even in long days. To this hypothetical but plausible substance Harder gives the name metaplasin.

Papers published from Harder's laboratory since 1940 support the hypothesis that leaf shape is affected by a diffusible substance, the production of which is determined by length of day. Thus Meyer (1947) carried out the following experiment with plants of *Sedum kamtschaticum*. She decapitated plants and removed all leaves but one; and she studied the leaf shape on a regenerating shoot exposed entirely to long days. The shoot produced typical long-day leaves, illustrated in Fig. 7*a*. In other decapitated plants, the single remaining leaf on the main stem was exposed to short days, while the regenerating shoot was exposed to long days. Under these circumstances the hypothetical metaplasin is believed to flow upwards from the single leaf exposed to short days into the regenerating shoot, with the result that leaves on the regenerating shoot do not have a typical long-day shape, but show a transition from a short-day shape below to a long-day shape above (Fig. 7*b*).

Stephens (1945), in the course of a genetical analysis of leaf shape in cotton (which displays very marked heteroblastic development), found that the length of day affected the rate of change of leaf shape from node to node. In one strain (*JX*) of *Gossypium herbaceum*, grown in short days, the sinus at the base of each successive leaf becomes deeper until it reaches a maximum depth; whereas if the strain is grown in conditions of increasing day length the sinus depth in successive leaves first deepens, then becomes more shallow, and then deepens again.

Sen Gupta & Santosh (1947) have demonstrated that the amount of dissection of leaves of *Sesamum orientale* is a function of the photoperiod in which the leaves have developed. Under short days (10 hr.) all leaves had entire margins; under long days (16 hr.) only 42% of the leaves had entire margins: the remaining 58% were more or less dissected. These examples are sufficient to indicate that leaf shape in some plants reacts in a consistent way to length of day. Since most plants in cold-temperate latitudes produce leaves from spring to autumn it is worth investigating whether the annual rhythm of leaf shape, found in plants as varied as ivy and dandelion, is due in part to the annual rhythm of length of day. For instance, a succession of leaf shapes in *Taraxacum officinale* noticed by Griffiths (1924) is consistent with the hypothesis that dentate leaves are produced in spring and autumn, and relatively entire leaves in the summer. It must not be supposed that all manifestations of heteroblastic development are likely to be accountable to length of day: there is ample evidence that other factors are concerned, for in some plants (e.g. *Daucus carota*) heteroblastic development follows the same course whether the plants begin their life cycle in long or short days.

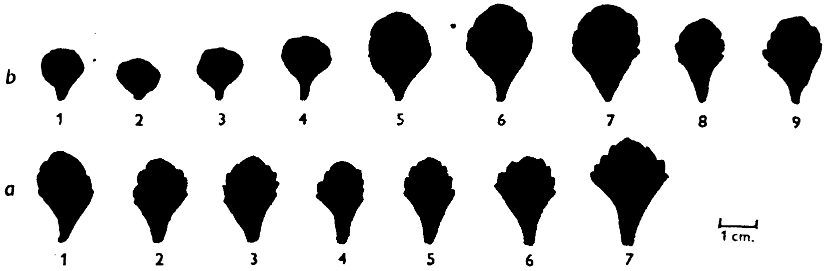


Fig. 7 (*a*). Successive leaves on a regenerating shoot of *Sedum kamtschaticum*. The shoot was attached to a decapitated plant from which all leaves but one were removed. The whole plant was exposed to long days, and the leaves had the typical long-day form.

Fig. 7 (*b*). Successive leaves on a regenerating shoot of *Sedum kamtschaticum* treated as for (*a*), but in which the single leaf left on the plant was exposed to short days, while the regenerating shoot was exposed to long days. In this experiment the lower leaves had the typical short-day form, and there was a gradual transition in shape from leaf to leaf to the typical long-day form. (Drawn from Meyer, 1947.)

This catalogue of effects of environment on leaf shape could be extended; but enough has been said here, for this essay is not intended to be an exhaustive survey of the literature of leaf morphogenesis. Many of the effects (e.g. those found among water plants) have been well summarized already; and others (e.g. the curious effects of hydrostatic pressure described by Pearsall & Hanby (1926)) cannot yet be integrated into a general hypothesis of leaf development.

The foregoing examples are sufficient to show that leaf shape is susceptible to external factors and may change from node to node in a regular way (as in heteroblastic development) under the influence of regular changes in some external factor. Changes in leaf shape may therefore reflect changes in the external environment; but there is ample evidence that the environment does not have exclusive control of leaf shape, and that heteroblastic development is not always accompanied by obvious changes in the environment. Some of this evidence will now be reviewed. In these instances it must be supposed (since causation of some sort has to be assumed) that leaf shape is affected by internal factors or by the shifting of some internal equilibrium. Goebel (1908) described how the shoots from a sprouting potato tuber produce successive leaves of different shapes, each more pinnate than its forerunner. If the tuber is cut into small pieces, each with one 'eye', and shoots are produced from these pieces, it is observed that there is no immediate succession of different leaf shapes; instead, as many as thirty simple or one-pinnate leaves are produced. Goebel attributed this to poor nutrition; and his hypothesis is supported by the work of Doposcheg-Uhlár (1911), who found that shoots from isolated palmate leaves of *Begonia carolineaefolia* bore simple and not palmate leaves in the first season. In 1914 Lundegårdh published observations on the leaf shape of *Ipomoea learii*, which indicate that a simple hypothesis depending on carbohydrate nutrition will not always account for heterophylly. In the genotype which Lundegårdh used there was a gradual transition in leaf shape from node to node, beginning at the base with entire leaves, and ending toward the apex with three-lobed leaves; a transition similar to that shown in Fig. 1*b*. This transition is repeated on each lateral branch, indicating, as Lundegårdh suggested, 'daß der Wechsel aus inneren Gründen erfolgt'. When cuttings of *I. learii* were taken, the leaf form of the leaves on a first order lateral produced on these cuttings depended upon the position of the lateral: if it was at the base, the lateral produced juvenile leaves with a gradual transition to adult leaves; if it was at the top of the cutting, the lateral produced adult leaves from the first node (Fig. 8). Lundegårdh speculated

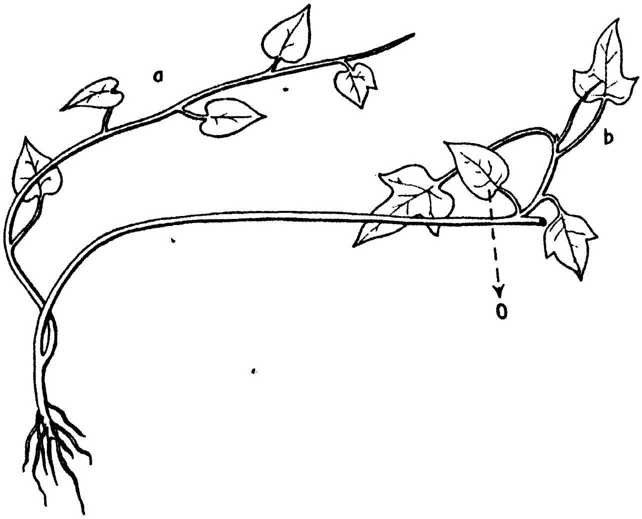


Fig. 8. A sucker of *Ipomoea learii* which has been cut off and rooted. The stem carries one leaf (*o*) which is a juvenile form. The cutting produces two lateral shoots, one at the base (*a*), the other at the tip (*b*). Shoot *a* carries four juvenile leaves and one (the fifth) transitional leaf. Shoot *b* carries three leaves, all of the adult form. (Redrawn from Lundegårdh, 1914.)

that this position effect might be due to 'growth enzymes' which change as they ascend the stem: an ingenious prediction considering the time at which it was made.

Another example of the effect of morphological position on leaf shape comes from the data of Kranz (1930) on the complex problem of leaf form in the ivy. Earlier observers of leaf shape in ivy (e.g. Furlani, 1914) believed that the youth form—a 5-lobed leaf—was a response to damp and shady conditions. However Tobler, in his monograph on ivy (1912), disputed this opinion, and Kranz (1930) re-investigated the leaf form of ivy. He showed that successive leaves of ivy pass through a long transition of forms before the shape becomes constant. The youth form is 5-lobed, intermediate forms are 3-lobed, and adult forms are ovate. Ivy is undoubtedly heterozygous for leaf shapes and there are many variants of these forms. A transition in anatomy accompanies the transition in shape; youth forms have comparatively large cells and one or two layers of palisade tissue; adult forms have comparatively small cells and three layers of palisade tissue. The transition in any one plant may take years to accomplish, and intermediate forms persist so long that they are sometimes thought to be garden varieties.

Kranz grew cuttings under different conditions of light and moisture: shapes of the leaves produced were unaffected by these different conditions. He also grew cuttings from stems bearing juvenile leaves and from stems bearing adult leaves. Lateral shoots on these cuttings produced leaves similar to the leaves subtending the shoots. From his experiments Kranz was justified in concluding that leaf shape in ivy is a function of the age of the plant and is independent of the external environment; and that the shape of leaves produced on cuttings depends on the part of the plant from which the cuttings are taken, and not upon the external conditions under which the cuttings are grown. The present writer has made observations on the leaf form of *Passiflora* which lead to the same conclusions.

It is clear, therefore, that internal as well as external factors affect leaf shape, and that in many (and perhaps most) plants the succession of leaf shapes from node to node depends on the age of the plant and the position in which the leaves arise. Effects of age and position are, of course, due to internal conditions which may be analysed in terms of cell chemistry. One method of analysis is to study the concentration and distribution of growth-promoting substances in leaves; another method is to disturb the fields of growth in leaves by operations on the plant. Such operations have been criticized on the grounds that they produce teratological responses, as indeed many of them do; but provided the teratological responses can be interpreted with confidence, the criticism is not valid.

As to the first method of analysis, no work except that of Harder and his colleagues has been published up to the present on the relation between growth-promoting substances and leaf shape, though there are some data on the distribution of auxin in leaves in relation to growth rates of different parts. Thus Avery (1935) extracted auxin from parts of tobacco leaves by diffusion into agar, and found that there was a marked gradient in auxin concentration in a young leaf from low values at the tip to high values at the base; these values approximately correspond to the regions of low and high growth rate represented in Fig. 4. Delisle (1938) showed that the peak of auxin production in *Aster novae-anglicae* is reached when the leaf is 5–8 mm. long, which is the stage of growth at which cell division ends and cell elongation begins. Auricles arise at the base of the leaf comparatively late and it is suggested by Delisle that this delayed localized growth is associated with a persisting high auxin concentration in this region.

The second method of analysis, the disturbance of fields of growth by operations on the plant, has shown that the enlargement of a leaf may be affected by the growth substances produced from neighbouring leaves. Three examples illustrate this point. In a series of experiments on leaf development in *Solidago sempervirens*, Goodwin (1937) showed that the removal of the fifth leaf when it is about 3 cm. long increased the growth rate of the sixth leaf, though it did not affect its final size. This stimulation of growth rate was entirely suppressed by the continued application of auxin to the cut stump of the fifth leaf. Auxin suppresses the growth rate of the leaf above and reduces its final size by reducing cell size. If leaf size is affected by the neighbouring leaves, leaf shape may be affected too; at present there are no experiments which bear on this question.

A second example of the effect on leaves of unbalancing internal conditions is provided by the experiments of Avery (1934) on topping and suckering tobacco plants. He reports that the effect of topping plants is to prolong the growth of the upper leaves which, by cell expansion only, may show an increase of 32% in area.

A third example is from the work of Wolf & Gross (1937), also on topped tobacco plants. These workers found that in normal tobacco plants there is an increase in the ratio of length to breadth from leaf to leaf up the stem; the ratio is about 1·8 for the first leaf and rises to about 2·2 at the twentieth leaf. Plants decapitated above the ninth leaf show a large increase in leaf area in all leaves except the lowest, but do not show any consistent change in shape from leaf to leaf. The physiological effects of topping are likely to be twofold: to remove a 'sink' for water and inorganic salts, and at the same time to remove a 'source' for auxin. It is not known whether either, or both, of these factors cause the increase in leaf area and the change in leaf shape.

Experiments on the artificial application of auxin to produce teratological forms of leaves may lead to a better understanding of the part played by growth substances in controlling leaf shape. Thus both M. & R. Snow (1937) and Laibach & Mai (1936) obtained evidence that abnormally high concentrations of auxin cause the fusion of parts in leaf initials. M. & R. Snow obtained fused leaf initials in *Epilobium* and lupin, and Laibach & Mai obtained entire tomato leaves. In complementary experiments to this Skoog (1935) destroyed auxin in the growing points of *Pisum* plants by X-rays, and thereby stimulated the development of lateral shoots.

(4) *The genetical control of leaf shape*

There is ample evidence that leaf shape is controlled by genes. Notable contributions to the genetic analysis of leaf shape have been made by Sirks (1932) on *Phaseolus*, Imai (1930) on *Ipomoea*, and by several workers, whose results are discussed below, on *Gossypium*. It will be sufficient here to cite examples from two plants, *Tropaeolum* and *Gossypium*.

Whaley & Whaley (1942) observed the enlargement of leaves in three strains of *Tropaeolum*, differing in two pairs of alleles for leaf shape: *T. majus*, with entire leaves (genotype denoted by **LU** or **Lu**), *T. peltophorum* var. *fimbriatum*, with 3-lobed leaves (genotype denoted by **lU**), and the double recessive (genotype **lu**) which has 5-pointed leaves. The leaves of all three genotypes are 5-pointed when young. Leaves carrying **LU** change shape during development in the manner described by Smirnov & Zhelochovtsev (1931) (Fig. 3*b*). Leaves carrying **lu** do not change shape noticeably during development. The differences in shape between leaves carrying **LU** and leaves carrying **lu** are summarized by the relative growth ratios along lines corresponding to *AO* and *BO* in Fig. 3*b*. The value of *k* (see p. 157) for the genotype **LU** is about 1·3, and for the genotype **lu** is about 1·0.

The genetics of leaf shape is not often as simple as this, and the leaves of *Gossypium* provide what is probably a more typical example. Studies in the genetical control of leaf shape in cotton, made by Hutchinson (1934), Silow (1939), Hammond (1941) and Stephens (1944, 1945), have disclosed that leaf shape is controlled by several multiple alleles, which operate by producing differential growth rates in different parts of the leaf. For instance, in *G. hirsutum* the mutant gene **okra** produces a longer leaf, with narrower lobes and deeper sinuses, than the normal gene. Hammond found that these effects of **okra** could be attributed to the following simple developmental causes: the relatively longer leaf is the result of more cell divisions orientated on the long axis of the leaf; the relatively narrower lobes are the result of (i) initially narrower lobes in the primordium, and (ii) a higher relative growth ratio between lobe length and lobe breadth; the relatively deeper sinuses are the result of (i) delayed appearance of lateral lobes in the young primordia, and (ii) a higher initial relative growth ratio between the two dimensions (*a*) lobe length and (*b*) the distance from sinus base to petiole. Hammond's results for *Gossypium*, unlike Whaley's for *Tropaeolum*, indicate that differences in leaf shape between genotypes are established in the primordia. Similar early determination of leaf shape is reported by Avery (1933), Delisle (1938) and Smith (1934).

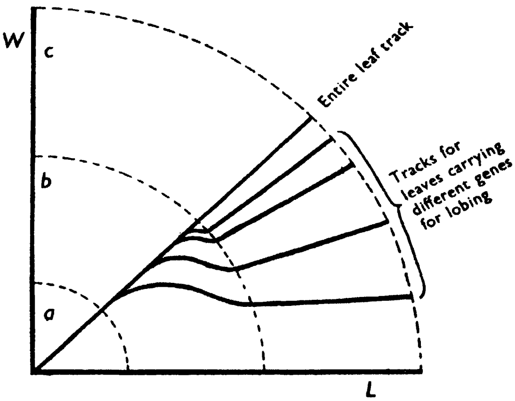


Fig. 9. Diagram illustrating Stephens's hypothesis of leaf shape organization in *Gossypium*. Ordinates (*W*) are logarithms of lobe width, abscissae (*L*) are logarithms of leaf length (median lobe). The lines are idealized regressions of *W* on *L*. (From Stephens, 1944.) For further explanation, see text.

Hammond extended her studies to cover the effects of the gene **okra** on the transition in shape between leaves on the first five nodes of *Gossypium hirsutum*. She found that leaves at the first node are almost alike in shape, whether they are homozygous for **okra** or not. Only at the second node does the gene **okra** begin to affect leaf shape, and even at the fifth node it does not exert its full effect.

Stephens (1945) carried the genetical analysis of leaf shape in *Gossypium* further still. He used the allometry formula to analyse the shapes at maturity of successive leaves on strains of cotton carrying one or other of the alleles for leaf shape. He found, in brief, that each allele acts on heteroblastic development by setting the change of leaf shape from node to node on a specific developmental track. He summarizes this by postulating that the young cotton plant, as it grows, passes through three phases of leaf development (Fig. 9): phase *a*, in which it produces entire leaves, phase *b* in which it produces leaves transitional between entire and lobed, provided genotypes for lobed leaf are present; and phase *c*, in which the lobed leaf is stabilized in one or other of several alternative patterns, depending on the genotypes. These developmental tracks summarize the course of heteroblastic development under the influence of different alleles. The appearance of a sinus, according to Stephens, is a consequence of the formation of a 'sinus field' of restricted cell division; when leaves on successive nodes appear more and more deeply dissected, it indicates that the sinus fields develop earlier and earlier in the ontogeny of the leaves.

HETEROBLASTIC DEVELOPMENT AND PHYSIOLOGICAL AGE

It may seem somewhat artificial to isolate the study of leaf shape from other contemporary problems in morphogenesis. But there is some reason to suppose that leaf shape has a special significance, in that the rate of change of leaf shape from node to node (i.e. the rate of heteroblastic development) may be a measure of the physiological age of the plant. The existing evidence for this view is examined in the following paragraphs. Subsequent papers in this series will contain experimental evidence bearing on the relation between leaf shape and physiological age.

There is clearly no single cause behind the phenomena of heteroblastic development. Some evidence indicates that leaf shape is a response to environmental conditions, such as carbohydrate balance, moisture, or length of day; other evidence indicates that leaf shape is determined by what are vaguely called 'internal factors' which are correlated with position on the plant and the age of the node from which the leaf arises. Even within the same species some genotypes show striking heteroblastic development and others do not; however, there seem to be few flowering plants in which there is not some consistent change in leaf shape, or in relative petiole length, from node to node. The general occurrence of these changes in shape which accompany ageing, inevitably leads to the question—Is there any relation between the rate of change in leaf shape in successive leaves and the physiological age of the plant?

Physiological age is an ill-defined, but none the less, evident concept of plant development. It is ill defined because hitherto there has not been any way to measure it quantitatively.* It is evident, because a great amount of practical knowledge about the vegetative and flowering phases of plants, the striking of cuttings from different parts of plants, and so on, is based on the assumption that plants of the same time-age may be in very different stages of maturity. It is a concept quite consistent with the evidence of plant physiology; for instance, Richards (1934) has drawn attention to the fact, revealed by experiments by Gregory and himself on barley, that the physiological behaviour of successive leaves is a function not only of the age of each leaf, but also of the age of the shoot from which the leaf arose. With plants, more easily than with animals, it is possible to separate experimentally time-age from physiological age, for maturity can be greatly delayed by some treatments and greatly accelerated by others. On these considerations it is worth while to examine the possibility of defining and measuring physiological age in plants.

Published work on the cotton plant provides an illustration of the possible relation between heteroblastic development and physiological age. The time of flowering in cotton usually corresponds to the appearance of a 'climax' shape of leaf. In varieties with lobed leaves there is a clear connexion between early flowering and the development of lobes on an early node: cultural conditions which hasten the one also hasten the other. Stephens (1944) states that the duration of juvenile foliage is adjusted to the duration of the normal pre-flowering period, and that heteroblastic development proceeds much more quickly on early flowering than on late flowering types. This confirms the work of Krenke, who published in 1933 data on the rate of change of leaf dissection from node to node in early and late types of cotton. Krenke found also that both time of flowering and the rate of change of leaf shape are dependent on the time of sowing of the cotton; and this observation is confirmed by Stephens (1945).

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* Benedict (1915) claimed that he could estimate 'senility' in *Vitis vulpina* and other woody perennials from the density of venation in leaves, which he measured as the number of vein islets per unit area. He published a large number of data, which have been analysed by the present writer. Considering the inevitable variability of biological material, these data show unexpectedly high correlations (e.g. $r=0{\cdot}96$) between the number of annual rings at the base of the vine and the number of vein islets per unit area, of a typical fully expanded leaf. This work was severely criticized by Ensign (1921), but Ensign contradicted rather than disproved Benedict's assertions. Although it is unlikely that the relation between leaf venation and age is as simple as Benedict believed, it would be worth while to re-examine Benedict's claims.

The evident parallelism in cotton between the stage of leaf shape reached, the stage of maturity (as determined by the time of flowering) and the node on which the first flowers are borne, led Krenke and his collaborators (1940) to make a survey of variability in leaf shape and to put forward a 'theory of cyclic ageing and rejuvenescence' in plants. Krenke's hypothesis is based on, and purports to receive its inspiration from, dialectical materialism. It contains a number of dubious propositions; it rests on slender evidence; and it does not deal adequately with data which do not conform to the hypothesis. Nevertheless, it is a bold and attractive idea, which is proving to be a suitable working hypothesis for experiments in morphogenesis. During the last two years the present writer has repeated many of Krenke's observations. Some have been confirmed; others cannot be confirmed. The following paragraphs contain a summary of such parts of Krenke's hypothesis as have stood up so far to the present writer's preliminary investigations, together with some of the data on which the hypothesis stands.

The data are essentially studies in the rate of change of leaf shape, from node to node (using the sort of criteria illustrated in Fig. 2), together with studies in physiological characters which accompany ageing. Some of the changes are cyclic (e.g. Fig. 2*c* and *d*). Others are not cyclic (e.g. Fig. 2*a*, *b* and *d*). Krenke postulated that these changes, and in particular the cyclic changes, are a measure of the physiological age of the plant.

Krenke's general hypothesis may be summed up by a diagram (Fig. 10) and the following comments upon it.

On the abscissae are plotted nodes, numbered from the base. Successive nodes are regarded by Krenke as units in a developmental scale, as distinct from a time scale. On the ordinates are plotted values for the criterion of physiological age. The criterion chosen depends on the plant: it has to be a characteristic which can be expressed quantitatively and which passes through a cycle or a progressive change from node to node. Suitable criteria are the amount of dissection of the leaf (cotton), the relative length of petiole and leaf blade (beet), the number of pinnae (*Dahlia*), the ratio of length to breadth (*Polygonum*), or even the length of internodes (*Morus*). Flowering usually follows the climax value of the criterion (Fig. 10*c*), and the right-hand tail of the curve (*de*) may not always be present.

The curve *abcde* is, according to Krenke, a measure of the cycle of physiological ageing. The physiological age of any node is measured by the value of the criterion at that node. External conditions which hasten maturity also increase the slope of the ascending part of the curve (*ab*), and the climax value (*c*) is reached at a relatively early node. External conditions which delay maturity also decrease the slope of the ascending part of the curve (*ab*), and the climax value (*c*) is deferred to a relatively late node. This assertion is supported by data published by Krenke (1940) on *Morus*, and by Belskaya (1940) and Minina (1940), on sugar-beet. In sugar-beet the normal succession of leaf shapes is as shown in Fig. 1*c*, and expressed quantitatively in Fig. 2*d*. Under heavy manuring the climax type of leaf occurs at a later node than it does in the controls. Fig. 11 *a* and *b* illustrates representative

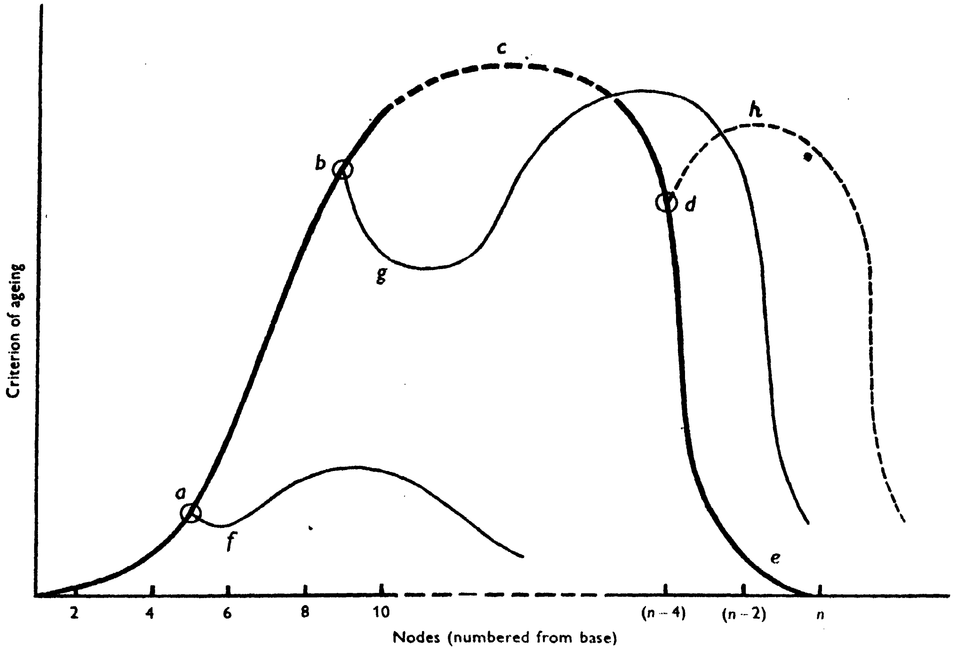

Fig. 10. Diagram to illustrate Krenke's hypothesis of cyclic ageing and rejuvenescence in plants. For explanation, see text.

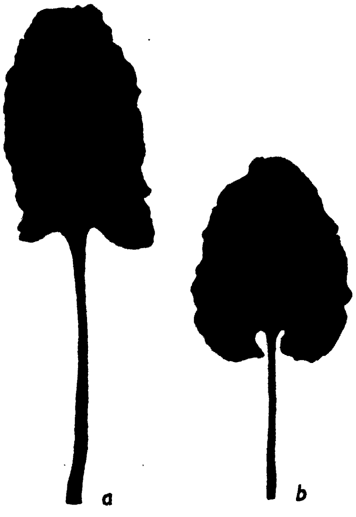

Fig. 11. Two sugar-beet leaves. Both are 16th leaves and both are about the same time-age. Leaf *a* is from a heavily watered, late maturing plant, and leaf *b* is from a sparsely watered, early maturing plant. On the criteria of the ratio of petiole length to blade length, together with the angle between the petiole and the leaf blade, leaf *a* is physiologically younger than leaf *b*. (From Minina, 1940.)

leaves, from corresponding nodes of two sugar-beet plants which have received high- and low-water treatments respectively. Both leaves are the same time-age, but on comparison with the series in Fig. 1*c* it is clear that on the criteria of leaf shape (petiole/blade ratio and angle between blade and petiole) leaf *b* is physiologically older than leaf *a*. There is evidence of a parallelism between these leaf criteria and the chemical condition of the plant; for instance, using some of Belskaya's data the present writer calculated a correlation of $r = 0{\cdot}914 \pm 0{\cdot}182$ between the shape of the last leaf to develop and the sugar content of the root. Krenke suggested further, though his evidence for this is meagre, that this correlation between the criteria of physiological age and maturity is true not only within varieties but between varieties, so that plants may be selected for early maturity at the seedling stage, because they will show, even in the first two or three nodes, a more precocious development of the criteria of physiological age. This suggestion of Krenke is illustrated by Fig. 12, taken from the work of Dubrovitskaya (1940), which shows the leaf development of two races of *Trachyspermum copticum* L.—an early race and a late race. In this example the rate of change of leaf shape is greater in the early than in the late variety.

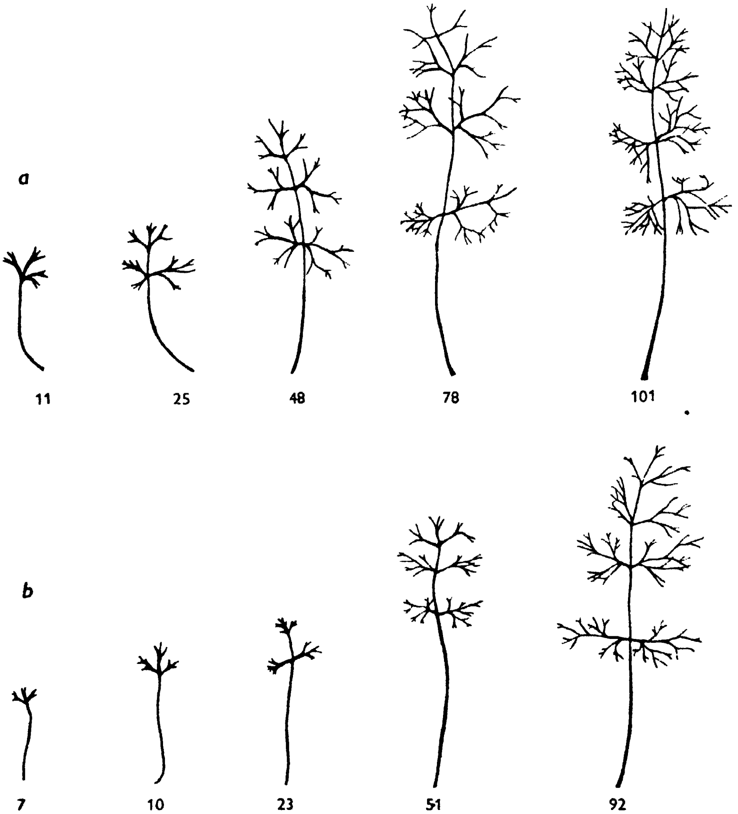

Fig. 12. Early maturing (*a*) and late maturing (*b*) varieties of *Trachyspermum copticum* L. The numbers under the leaves refer to the number of segments. Note that a higher rate of heteroblastic development is already apparent at the second leaf on variety *a*. Krenke asserts that this higher rate is a diagnostic character of early maturing varieties. (Drawn from data of Dubrovitskaya, 1940.)

A further inspection of Fig. 10 brings out another aspect of Krenke's theory of cyclic development. Krenke maintained that the shooting of a lateral branch is always accompanied by some rejuvenescence. Therefore he believed that the first leaf on a lateral shoot is physiologically younger, even though it is older in time, than the leaf subtending the lateral shoot. The amount of this rejuvenescence is revealed by the criteria of physiological age on the lateral branch. Suppose the criterion were the amount of dissection of the leaf; then the first leaf on a lateral from node 9 (Fig. 10*g*) should be similar to, but slightly *less* dissected, than the leaf at node 9 on the main stem (*b*); and the first leaf (*h*) on a lateral from node $(n-4)$, after the climax form has been passed, should be similar to, but slightly *more* dissected than, the leaf at node $(n-4)$ on the main stem (*d*). This Krenke found to be the case, and the present writer has sometimes, though not invariably, obtained evidence which is consistent with Krenke's hypothesis (Fig. 13). Lundegårdh's

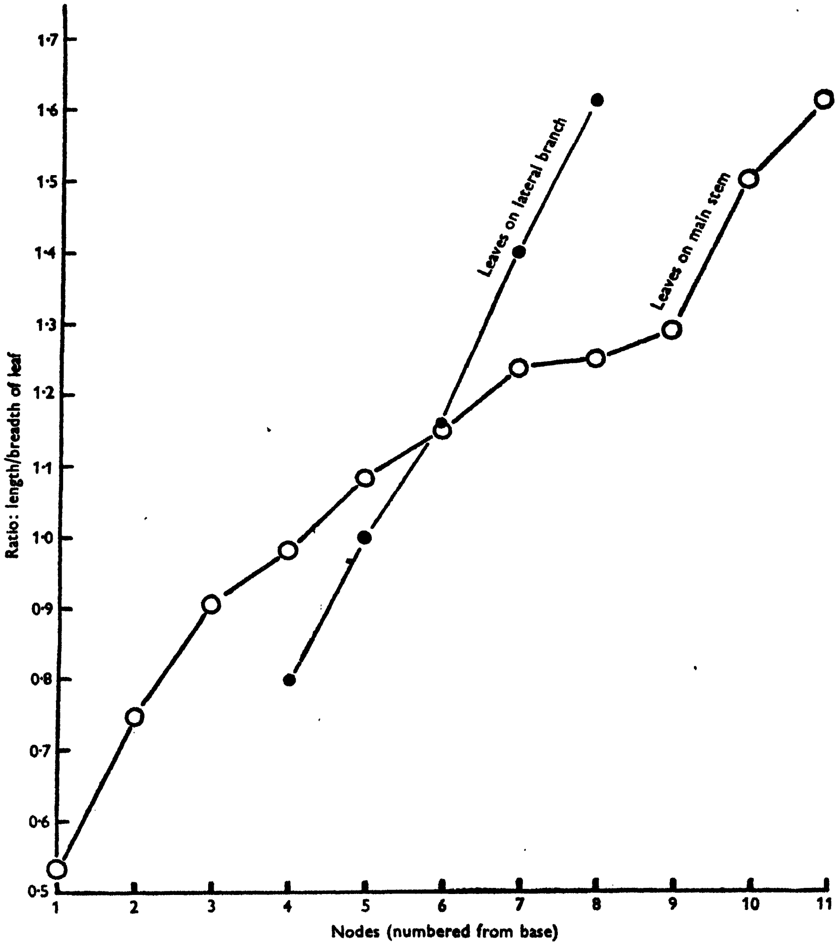

Fig. 13. The ratio of length to breadth of leaf blade in *Polygonum cuspidatum*, plotted against node of insertion. —○— values for leaves on the main stem. —●— values for leaves on a lateral arising from the fourth node. For further explanation, see text. (Data original.)

observation on *Ipomoea* (see Fig. 8) might be regarded as an illustration of the rejuvenescence of laterals, and Hammond states (1941) that in cotton the transition in leaf shape from node to node on the main stem is repeated, though more irregularly, on the laterals. Krenke considered that the amount of rejuvenescence to be found in a lateral depends on the physiological age of the node from which it arises. Rejuvenescence from a physiologically young node (e.g. node 5, see Fig. 10) is feeble, and the first leaf on a lateral from this node (*f*) would scarcely differ in shape from the subtending leaf (*a*). Some of Krenke's evidence for these views is summarized in the following table:

Leaf dissection in Gossypium herbaceum (*data from Krenke*, 1933–5)

Values of correlation coefficients (r) for the degree of dissection of leaves and other characters in cotton.

	r	Standard error
(1) Dissection of leaf and node of insertion:		
(*a*) Ascending curve of dissection (i.e. *ab* in Fig. 10)	0·773	±0·018
(*b*) Climax region of curve (i.e. *c* in Fig. 10)	0·027	±0·064
(2) Dissection of leaf on main branch and dissection of first leaf of corresponding lateral	0·895	±0·053
(3) Dissection of first leaf on lateral and node of origin of lateral	0·632	±0·042

CONCLUSION

The object of this essay is to define the problems which lie in the study of leaf shape and heteroblastic development. These problems may be enumerated as: (*a*) the expression of differences in shape between leaves in terms of developmental anatomy; (*b*) the discovery of cytochemical differences underlying these differences in shape; (*c*) the elucidation of anatomical and chemical changes which cause the changes in leaf shape from node to node typical of so many plants; (*d*) the determination as to whether this rate of change of leaf shape is (as Krenke suggests) a measure of the rate of ageing of the plant. None of these problems can be solved with the data at present available, though the data are sufficient to show that leaf shape is of more than trivial interest to botanists. Observation and experiment along traditional lines will undoubtedly go some way toward solving the problems; but leaf shape is the product of tissue organization, and there is no reason to expect (and some justification not to expect) that a knowledge of organization at the level of the cell will explain organization at the level of the tissue. Sinnott (1940) has shown how the development of *Cucurbita* fruits takes place as though the size and shape of the whole fruit were controlled independently of cell size and shape, by some organizer which co-ordinates the behaviour of whole groups of cells. If Sinnott's view is correct, then the causes of leaf shape and heteroblastic development must be sought not only in cell chemistry, but in a system of organization which transcends individual cells.

SUMMARY

1. Commonly among flowering plants there are transitions from youth to adult forms of leaves, visible as changes in leaf shape from node to node, and called by Goebel heteroblastic development.

2. Heteroblastic development can be expressed quantitatively and therefore its rate of change from node to node can be measured.

3. Leaf shape in some plants is affected by light intensity, possibly operating through carbohydrate metabolism, and by mineral nutrition. In other plants (e.g. species of *Morus*, *Kalanchoë*, *Sedum*, *Gossypium* and *Sesamum*), leaf shape and heteroblastic development are dependent to some extent upon length of day.

4. In some plants no correlation has been discovered between leaf shape and external conditions. In these plants (e.g. species of *Ipomoea* and *Hedera*) leaf shape is a function of the age of the plant and the position on the plant at which the leaf arises. Heteroblastic development is presumably controlled by unknown internal factors.

5. Both leaf shape and rate of heteroblastic development are influenced by genes. For *Gossypium* the effects of some specific genes on heteroblastic development have already been worked out.

6. The hypothesis of Krenke, that the rate of heteroblastic development may be used to measure physiological age, is examined in the light of Krenke's own data and some of the present writer's observations.

7. Some of the problems which lie in the study of leaf shape and heteroblastic development are defined.

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REFERENCES

Abbé, E. C., Randolph, L. F. & Einset, J. (1941). The developmental relationship between shoot apex and growth pattern of leaf blade in diploid maize. *Amer. J. Bot.* **28**, 778.

Åberg, B. (1943). Physiologische und ökologische Studien über die pflanzliche Photomorphose. *Symbolae botanicae Upsalienses*, **8**, 1, Uppsala.

Avery, G. S. (1933). Structure and development of the tobacco leaf. *Amer. J. Bot.* **20**, 565.

Avery, G. S. (1934). Structural responses to the practice of tobacco-topping. *Bot. Gaz.* **96**, 314.

Avery, G. S. (1935). Differential distribution of a phytohormone in the developing leaf of *Nicotiana*.... *Bull. Torrey Bot. Cl.* **62**, 313.

Belskaya, T. N. (1940). The diagnosis of efficient manurial treatment of sugar-beet. In *Theory of Cyclic Ageing and Rejuvenescence of Plants*, p. 59. Moscow (Russian).

Benedict, H. M. (1915). Senile changes in leaves of *Vitis vulpina* L. *Mem. Cornell Agric. Exp. Sta.* **7**, 271.

Bitter, G. (1897). Vergleichend-morphologische Untersuchungen über die Blattformen der Ranunculaceen und Umbelliferen. *Flora, Jena*, **83**, 223.

Brown, S. W. (1944–5). Studies of development in larkspur. 1. Form sequence in the first ten mature leaves. *Bot. Gaz.* **106**, 103.

Cushman, J. A. (1902). Studies of localized stages of growth in some common New England plants. *Amer. Nat.* **36**, 865.

Cushman, J. A. (1903). Studies of localized stages in some plants of the botanic gardens of Harvard University. *Amer. Nat.* **37**, 242.

Cushman, J. A. (1904). Localized stages in common roadside plants. *Amer. Nat.* **38**, 819.

Delisle, A. L. (1938). Morphogenetical studies in the development of successive leaves in *Aster*. *Amer. J. Bot.* **25**, 420.

Diels, L. (1906). *Jugendformen und Blütenreife in Pflanzenreich.* Berlin.

Doposcheg-Uhlár, J. (1911). Studien zur Regeneration und Polarität der Pflanzen. *Flora, Jena,* **102**, 24.

Dubrovitskaya, N. E. (1940). Diagnosis in the seedling stage of earliness in two races of *Trachyspermum copticum.* In *Theory of Cyclic Ageing and Rejuvenescence of Plants*, p. 77. Moscow (Russian).

Ensign, M. R. (1921). Area of vein islets in leaves of certain plants as an age determinant. *Amer. J. Bot.* **8**, 433.

Furlani, J. (1914). Zur Heterophyllie von *Hedera helix* L. *Öst. bot. Z.* **5**, 153.

Gertrude, M. T. (1937). Action du milieu extérieur sur le métabolisme végétal: métabolisme et morphogénèse en milieu aquatique. *Rev. gén. Bot.* **49**, 160.

Gessner, F. (1940). Beiträge zur Biologie amphibischer Pflanzen. *Ber. dtsch. bot Ges.* **68**, 2.

Goebel, K. (1898). *Organographie der Pflanzen,* 1st ed., p. 123. Jena.

Goebel, K. (1908). *Einleitung in die experimentelle Morphologie der Pflanzen.* Leipzig.

Goodwin, R. H. (1937). The role of auxin in leaf development in *Solidago*. *Amer. J. Bot.* **24**, 43.

GRIFFITHS, B. M. (1924). A note on the periodicity of leaf form in *Taraxacum officinale*. *New Phytol.* **23**, 153.

HAMMOND, D. (1941). The expression of genes for leaf shape in *Gossypium hirsutum* L. and *Gossypium arboreum* L. *Amer. J. Bot.* **28**, 124.

HARDER, R. & v. WITSCH, H. (1940). Über den Einfluss der Tageslänge auf den Habitus, besonders die Blattsukkulenz und den Wasserhaushalt von *Kalanchoë Blossfeldiana*. *Jb. wiss. Bot.* **89**, 354.

HUTCHINSON, J. B. (1934). The genetics of cotton. X. The inheritance of leaf shape in Asiatic *Gossypium*. *J. Genet.* **28**, 437.

IMAI, Y. (1930). A genetic monograph on the leaf form of *Pharbitis Nil*. *Z. indukt. Abstamm.- u. VererbLehre*, **55**, 1.

JACKSON, R. T. (1899). Localised stages in development in plants and animals. *Mem. Boston Soc. Nat. Hist.* **5**, no. 4.

KELLER, B. A. & POPLAVSKY, K. M. (1934). The changing of leaf shape in *Taraxacum officinale* by the action of physico-chemical factors on the rootstock. *Experimental Botany*, **1**, 7. Leningrad (Russian).

KRANZ, G. (1930). Zur Kenntnis der wechselnden Blattform der Efeus und ihrer Ursachen. *Flora, Jena*, **125**, 289.

KRENKE, N. P. (1933–5). A morphogenetical analysis of the cotton plant. *Phenogenetical Variability*, **i**, p. 419. (Russian.)

KRENKE, N. P. (1940). *Theory of Cyclic Ageing and Rejuvenescence of Plants*, p. 32. Moscow (Russian).

LAIBACH, F. & MAI, G. (1936). Über die künstliche Erzeugung von Bildungsabweichungen bei Pflanzen. *Roux Arch. Entw. Mech. Organ.* **134**, 200.

LUNDEGÅRDH, H. (1914). Experimentell-morphologische Beobachtungen. *Flora, Jena*, **107**, 433.

MAXIMOV, N. A. (1926). *The Plant in Relation to Water*. London.

MEYER, G. (1947). Über die photoperiodische Beeinflussbarkeit des Habitus von *Sedum kamtschaticum*. *Biol. Zbl.* **66**, 1.

MININA, E. G. (1940). The diagnosis of efficient irrigation of sugar-beet. In *Theory of Cyclic Ageing and Rejuvenescence of Plants*, p. 61. Moscow (Russian).

MOTHES, K. (1932). Ernährung, Struktur, und Transpiration. *Biol. Zbl.* **52**, 193.

ORSHANSKY, G. (1938). Seasonal dimorphism in *Ononis natrix* L. *Palestine J. Bot.* **1**, 233.

VON PAPEN, R. (1935). Beiträge zur kenntnis des Wachstums der Blattspreite. *Bot. Arch.* **37**, 159.

PEARSALL, W. H. (1927). Growth Studies. VI. On the relative sizes of growing plant organs. *Ann. Bot., Lond.*, **41**, 549.

PEARSALL, W. H. & HANBY, A. M. (1925). The variation of leaf form in *Potamogeton perfoliatus*. *New Phytol.* **24**, 112.

PEARSALL, W. H. & HANBY, A. M. (1926). Growth Studies. V. Factors affecting the development and form of leaves. *Ann. Bot., Lond.*, **40**, 85.

RICHARDS, F. J. (1934). On the use of simultaneous observations on successive leaves for the study of physiological change in relation to leaf age. *Ann. Bot., Lond.*, **48**, 497.

RICHARDS, O. W. & KAVANAGH, A. J. (1945). The analysis of growing form. In *Essays on Growth and Form*, p. 188. Oxford.

SCHÜEPP, O. (1945). Allometrie und Metamorphose. *Verh. naturf. Ges. Basel*, **56**, 261.

SEN GUPTA, J. C. & SANTOSH, K. P. (1947). Leaf heteromorphism and photoperiods in *Sesamum orientale* L. *Nature, Lond.*, **160**, 510.

SHULL, G. H. (1905). Studies in the development of *Sium cicutaefolium*. *Publ. Carneg. Instn*, **30**.

SIFTON, H. B. (1944). Developmental morphology of vascular plants. *New Phytol.* **43**, 87.

SILOW, R. A. (1939). The genetics of leaf shape in diploid cottons and the theory of gene interaction. *J. Genet.* **38**, 229.

SINNOTT, E. W. (1940). The cell-organ relationship in plant organisation. *Growth Supplement*, **77**.

SINNOTT, E. W. (1946). Substance or system: the riddle of morphogenesis. *Amer. Nat.* **80**, 497.

SIRKS, M. J. (1932). Beiträge zu einer genotypischen Analyse der Ackerbohne, *Vicia Faba* L. *Genetica*, **13**, 210.

SKOOG, F. (1935). The effect of X-irradiation on auxin and plant growth. *J. Cell. Comp. Physiol.* **7**, 227.

SMIRNOV, E. & ZHELOCHOVTSEV, A. N. (1931). Das Gesetz der Altersveränderungen der Blattform bei *Tropaeolum majus* L. unter verschiedenen Beleuchtungsbedingungen. *Planta*, **15**, 299.

SMITH, G. H. (1934). Anatomy of the embryonic leaf. *Amer. J. Bot.* **21**, 194.

SNOW, M. & SNOW, R. (1937). Auxin and leaf formation. *New Phytol.* **36**, 1.

SÖDING, H. (1934). Über die Bedingungen für die Entstehung der Sonnenblätter. *Ber. dtsch. bot. Ges.* **52**, 110.

SPORN, E. (1926). Einiges über Ideal-, Normal- und Individual-formen der Blätter. *Roux Arch. Entw. Mech. Organ.* **107**, 393.

STEPHENS, S. G. (1944). The genetic organisation of leaf shape development in the genus *Gossypium*. *J. Genet.* **46**, 28.

STEPHENS, S. G. (1945). Genetic survey of leaf shape in New World cottons. *J. Genet.* **46**, 313.

TOBLER, F. (1912). *Die Gattung Hedera*. Leipzig.

TUMANOV, J. J. (1932). Ungenügende Wasserversorgung und das Welken der Pflanzen als Mittel zur Erhöhung ihrer Durresistenz. *Planta*, 3, 391.

WHALEY, W. G. & WHALEY, C. I. (1942). A developmental analysis of inherited leaf patterns in *Tropaeolum*. *Amer. J. Bot.* **29**, 195.

WOLF, F. A. & GROSS, P. M. (1937). Flue-cured tobacco: a comparative account of structural responses induced by topping and suckering. *Bull. Torrey Bot. Cl.* **64**, 117.

WOLTERECK, I. (1928). Experimentelle Untersuchungen über die Blattbildung amphibischer Pflanzen. *Flora, Jena*, **123**, 30.

YAPP, R. H. (1912). *Spiraea Ulmaria* and its bearing on the problem of xeromorphy in marsh plants. *Ann. Bot., Lond.*, **26**, 815.

ZALENSKI, V. (1904). Materials for the study of the quantitative anatomy of different leaves on the same plant. *Mém. Polytech. Inst. Kiev*, 4, 1. (Russian.)

STUDIES IN THE MORPHOGENESIS OF LEAVES

II. THE AREA, CELL SIZE AND CELL NUMBER OF LEAVES OF *IPOMOEA* IN RELATION TO THEIR POSITION ON THE SHOOT

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(With 13 figures in the text)

CONTENTS

PAGE
1. Introduction 177
2. Summary of experimental procedure . . 179
3. Details of experimental procedure . . 180
    Material 180
    Cultivation and treatments . . . 181
    Collection of leaves 181
    Examination of leaves 181
4. Summary of data 183
    Morphology of leaves on control plants . 183
    Morphology of leaves from treated plants . 185
5. Discussion 188
6. Summary 191
References 192
Appendices 192

1. Introduction

In Maximov's book, *The Plant in Relation to Water* (1929), there is an important chapter on the effects of water supply on leaf structure. Two views set out in this chapter are still generally accepted, namely (i) that some xeromorphic characters, especially those depending on cell size in leaves, can be induced by subjecting plants to water shortage; and (ii) that 'the anatomical structure of individual leaves on one and the same shoot is, so to speak, a function of their distance from the root system' (Maximov, 1929, p. 332). This second view follows from the work of Zalenski (1904), who found on shoots of *Nicotiana rustica*, *Dactylis glomerata*, and other plants a consistent decrease in the size of epidermal cells from the lower to the upper leaves, accompanied by other signs of increasing xeromorphy. This increasing xeromorphy from the lower to the upper leaves of a shoot might be explained on either of two simple hypotheses: the upper leaves may be produced under comparatively drier climatic conditions than the lower leaves; or the upper leaves may be deprived of water during their growth by the lower leaves. Zalenski favoured the second of these two hypotheses and it is now widely believed that the gradient of cell size found in succeeding leaves on a shoot is the result of competition for water among the leaves, whereby the upper leaves are deprived of some water by the lower leaves. Such observations as there are on the water content, osmotic pressure, and suction force of leaves at different levels of insertion on the shoot are consistent with this hypothesis; but the most convincing evidence comes from a well-known experiment by Alexandrov, Alexandrov & Timofeev (1921). These authors grew plants of *Bryonia dioica* and *Ipomoea purpurea* as single long shoots, by removing lateral buds. In one particular example, when forty leaves had developed, they removed all the leaves. Examination of these leaves revealed the expected trend from leaf to leaf in cell size (measured in this instance as stomatal frequency). Subsequently the authors examined, from the same shoot, succeeding leaves (leaf 41, 42, etc.) which had developed in the absence of the first forty leaves. These leaves had cells no smaller than the cells in the twentieth leaf.

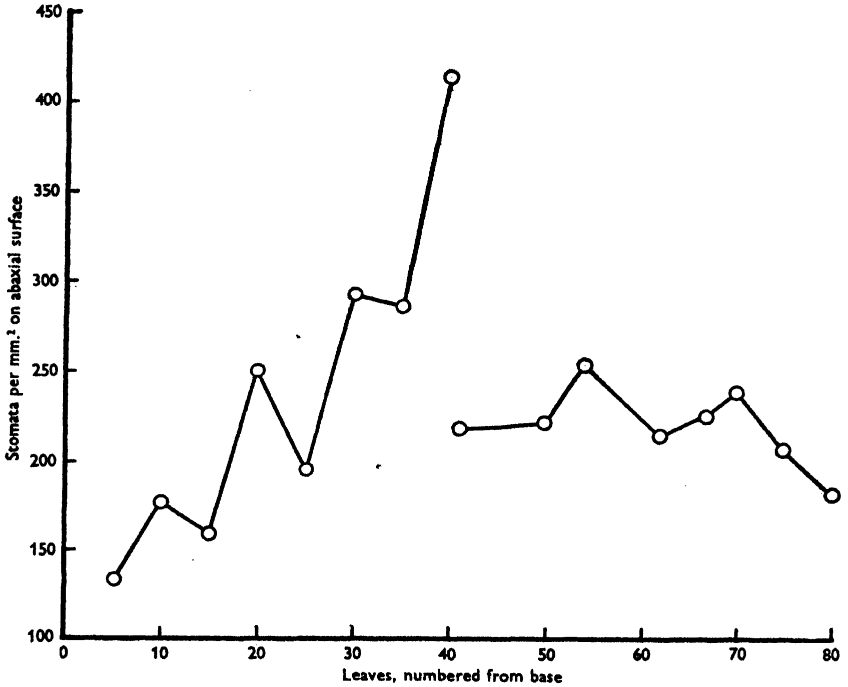

Fig. 1. Stomata per mm.² on the abaxial surface of *Ipomoea purpurea* (ordinates), and leaves numbered from the base (abscissae). Leaves 1–40 were removed. Data from Alexandrov *et al.* (1921), table 3.

The data are illustrated in Fig. 1. The authors concluded that the sudden change in cell size of upper leaves is due simply to the greater quantity of water available when the lower leaves have been removed, and they added that the change is less marked when the plants are grown in shade than when they are grown in sun.

There is no doubt that the facts, as described by Zalenski and many workers after him, are correct; but there is considerable doubt as to the interpretation commonly put upon these facts. In the first place the data of Alexandrov, Alexandrov & Timofeev, which are the mainstay of the interpretation, are misleading. These authors had no controls with which to compare their treated plants, and there is no doubt whatever that the striking change in cell size which follows cutting (e.g. the difference between the fortieth and the forty-first leaf in Fig. 1) is due in part to the fact that the upper leaves were cut off before they were fully expanded: it is leaf 40, not leaf 41, which is abnormal. In the second place, it has been shown by Goodwin (1937) and others that the growth of a young leaf is inhibited by auxin diffusing from the leaf below it; and if the leaf below is removed, the young leaf is released from this inhibition and grows more rapidly. It is possible, therefore, that the effects of removing lower leaves on the cell size of succeeding leaves above them may be due to interference with auxin gradients, and not to the release of additional water for the upper leaves. In the third place, there are no data from plant physiology to justify the belief that the lower leaves on a shoot do deprive the upper leaves of water.

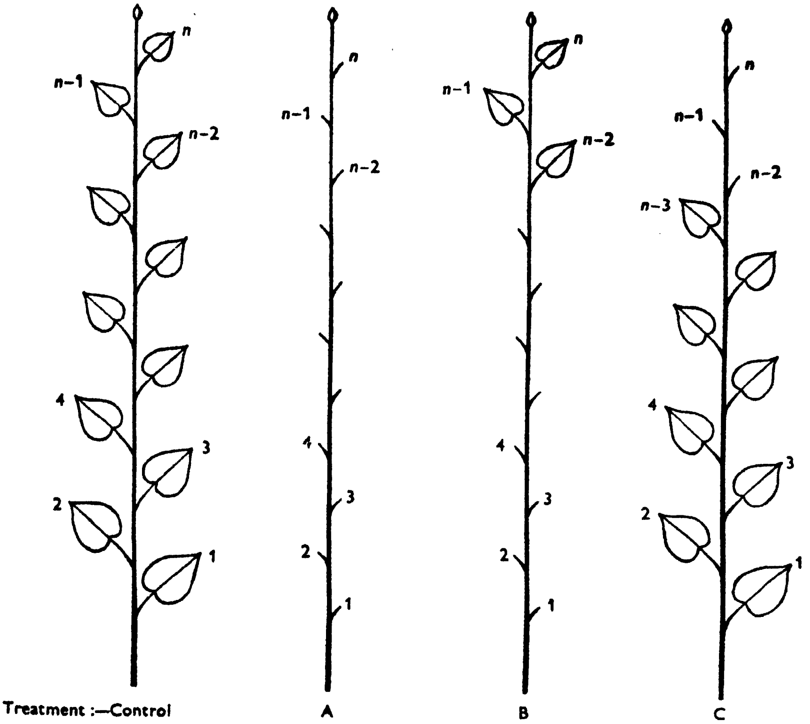

Fig. 2. Diagram to illustrate the four treatments applied to *Ipomoea*. For explanation see text, § 2.

On these grounds the author has re-examined the question of the relation between cell size in leaves and their level of insertion on a shoot. This re-examination is a necessary prelude to the author's studies in morphogenesis of leaves, the purpose of which was fully described in the first paper of this series (Ashby, 1948).

2. SUMMARY OF EXPERIMENTAL PROCEDURE

The experiments were carried out to analyse the effects of the lower leaves of a shoot on the morphology of the upper leaves, and in particular to examine the hypothesis that these effects are due to competition for water among the leaves.

Plants consisting of a single shoot were grown under dry and under damp conditions, and were subjected to the following four treatments which are illustrated in Fig. 2:

(i) *Control.* All leaves left on the shoot (Fig. 2, Control).

(ii) *Treatment A.* Leaves 1 to n were removed. Morphological studies were made on leaves $(n+1)$, $(n+2)$, $(n+3)$, etc. (Fig. 2A).

(iii) *Treatment B.* Leaves 1 to $(n-3)$ were removed. Morphological studies were made on leaves $(n+1)$, $(n+2)$, $(n+3)$, etc. and on leaf $(n-2)$ (Fig. 2B).

(iv) *Treatment C.* Leaves $(n-2)$ to n were removed. Morphological studies were made on leaves $(n+1)$, $(n+2)$, $(n+3)$, etc. and on leaf $(n-3)$ (Fig. 2C).

These treatments were applied on the hypothesis that: (i) if, on the one hand, water supply is the determining factor in leaf morphology, then leaf $(n+1)$ should respond to treatment B in the same way as to treatment A, and it should respond to treatment C in the same way as to the controls; (ii) if, on the other hand, a leaf hormone, or some similar influence, is the determining factor in leaf morphology, then leaf $(n+1)$ should respond to treatment B in the same way as to the controls, and it should respond to treatment C in the same way as to treatment A. Furthermore, if water supply is the controlling factor, the morphological response should be more pronounced under dry conditions than under wet conditions.

3. DETAILS OF EXPERIMENTAL PROCEDURE

Material

Three garden varieties of *Ipomoea* were used:

(i) *Ipomoea caerulea* Koen. ex Roxb, carrying normal dominant genes for leaf shape (Imai, 1930, fig. 1); to be called strain x.

(ii) *I. hederacea* Jacq. var. *grandiflora* Hort. carrying normal dominant genes for leaf shape, but differing from strain x for other genes; to be called strain y.

(iii) *I. hederacea* Jacq. 'purple stripe', carrying the incompletely recessive gene (or genes) 'cordate' (Imai, 1930, fig. 6); to be called strain z.

All three varieties are herbaceous annuals, and are twining plants.

Cultivation and treatments

Seed was sown on 14 April 1947, in 5 in. pots in a 'John Innes compost'. Eighty plants of each variety were raised, i.e. 240 plants altogether, one in each pot. Immediately after germination, and without transplanting, forty plants of each variety were put into one glasshouse (house 1) and forty into another glasshouse (house 2). The two glasshouses lie parallel to each other and 10 ft. apart, and they receive the same amount of light. Both houses received the same amount of heating, though they were not always at the same temperature. House 1 was kept as dry as possible and the plants in it were watered only sufficiently to maintain normal growth. The leaves frequently wilted for a short time during the day, and once during the experiment, when there were about six leaves on the plants, the soil in the pots in house 1 became so dry that the pots had to be soaked out in water. House 2, in contrast, was kept continually moist. A hot water-bath maintained the air at a high relative humidity, and the plants were watered every day.

In both houses the potted plants were labelled according to the treatments they were to receive, and were then arranged entirely at random, 9 in. apart, on the east side of each house. After thinning, there was a uniform population of seedlings, and by 11 May 1947, most of the plants possessed one or two leaves. On 11 May, and regularly thereafter, the dimensions of every leaf were measured. The measurements taken are illustrated by the broken lines in Fig. 3. The purpose of these measurements was to enable any changes in shape during enlargement to be followed, and so that there could be no doubt as to whether any particular leaf was fully expanded or not. All lateral buds were removed as they appeared. For the greater part of the experiment the plants in house 2 had significantly longer internodes, but did not have significantly more nodes, than the plants in house 1.

On 6 and 7 June, when plants in both houses had produced about ten leaves, the treatments illustrated in Fig. 2 were applied. The leaves were cut at the base of the petiole with surgical scissors, and preserved for examination. Since, at the time of operation, different plants had different numbers of nodes, the *n*th leaf referred to in Fig. 2 and §2 was not always the tenth leaf: it was the topmost leaf which had emerged from the terminal bud, and it varied from the ninth to the eleventh leaf.

After the treatment had been applied, the plants continued to grow and produced seven to eight new leaves on the single shoot. The dimensions of these leaves were regularly measured. On 26 and 27 June these leaves, and the leaves on the control plants, were removed for examination.

Collection of leaves

When leaves were cut from the plants for treatments A, B and C, the following operations were carried out:

(i) Small cuts were made on the margin of each leaf according to a code system, so that later on each leaf could be assigned to its node of insertion on the shoot.

(ii) The leaves were then cut from the shoot and immediately laid under a sheet of glass on ferro-prussiate blue-print paper, in order to provide a permanent record of the size and shape of the leaves.

(iii) The leaves cut from each shoot were then wrapped in a roll, bound with soft wire (tin-lead alloy fuse wire was used), labelled with the number of the plant, and preserved in a mixture of alcohol, water, and glycerine in the proportions 2:2:1.

(iv) At the end of the experiment, all the leaves from control plants, and the leaves which had appeared since the operations on treated plants, were coded as described in (i) above, cut from the plants, photographed on blue-print paper, and preserved in the alcohol-water-glycerine mixture for examination.

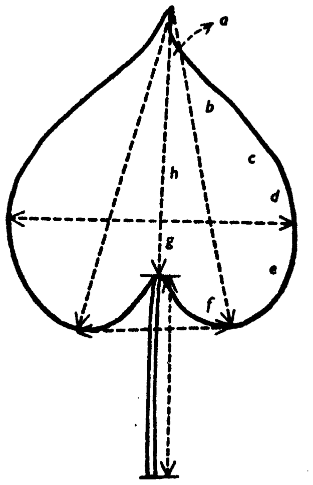

Fig. 3. Diagram to illustrate the measurements taken on growing leaves (broken lines), and the regions of the leaf sampled for cell size (*a*, *b*, . . ., *g*, *h*).

Examination of leaves

From the data collected on growth rates and sizes of leaves while still on the plants, it was possible to decide which of the preserved leaves were not fully expanded; data for area and cell size of these leaves were rejected. On all fully expanded leaves the following observations were made: (i) leaf area, (ii) number of epidermal cells per mm.^{2} on the adaxial surface of the leaf blade, (iii) total number of epidermal cells on the adaxial surface of the leaf blade, (iv) leaf shape. The last of these measurements, leaf shape, is not considered in the present paper; it is to be the subject of the third contribution to this series. Details of the other observations are as follows:

(i) *Leaf area.* Leaf areas were measured from the blue-print records, with a planimeter. Each measurement is the mean of two readings.

(ii) *Number of epidermal cells per mm.*^{2} Estimates of cell size were made from the adaxial epidermis, because it is easier to remove than the abaxial epidermis, and has fewer stomata. Pieces of leaf about 1 cm.^{2} in area were cut from the leaf, washed in 20% alcohol and then in water, and left in 10% nitric acid for 3–5 min. This treatment softens the tissues so that the epidermis can be stripped off with fine forceps. The strips of epidermis were transferred successively to 10, 30 and 50% alcohol, stained in Ehrlich's haematoxylin for about 3 min., washed in 50% alcohol, left for 3 min. in a mixture of equal parts of 50% alcohol and glycerine, and mounted in glycerine jelly.

Cell counts from epidermal cells prepared in this way were compared with counts from cells on the same leaf blade mounted without any treatment, in order to determine whether the treatment led to a measurable distortion in cell size. The value of 't' for a null hypothesis was 0·102, and of P was 0·8. The probability of any error from this source may therefore be neglected.

Cell counts were made with the aid of an Ehrlich eyepiece, with an adjustable square field. A ⅛ in. objective was used, and the area of the square field was 0·0324 mm.^{2} Since the epidermal cell walls are convoluted, it is difficult to estimate the number of incomplete cells in the field. Accordingly, only whole cells were counted, and a correction was added for incomplete cells. This correction was made by calculating the regression of half the number of incomplete cells on the square root of the number of whole cells in the field. The reason this form of regression was used is given in the Appendix. The regression is approximately linear, with a coefficient of 1·6491 ± 0·1005.

Cell counts were made from different regions of the same leaf blade in order to determine the reliability of estimates from small areas of leaf surface. Ten samples were taken from each of eight regions of the leaf blade (the regions marked *a*, *b*, *c*, ..., *h* in Fig. 3). The results for one cordate leaf (1) and one lobed leaf (2) are summarized in Table 1.

Table 1. *Variance ratio: between regions/within regions* (*F*), *and its significance* (*S*) *for epidermal cell counts on eight regions of the leaf blade of* Ipomoea; *also mean cell counts* (*m*) *and semi-fiducial limits at the* 5% *level for means of ten readings* (*L*)

Leaf		*F*	*S*	*m*	*L*
(1)	All regions	1·497	—	17·56	1·72
(2)	All regions	8·043	*S*	21·64	1·55
	All regions except tip, and base of sinus	2·142	—	20·58	1·58

It is concluded from this table that (i) apart from small local groups of cells at the tip of the blade and the base of the sinus, there is no significant variance from place to place on the epidermis; and (ii) cell counts in ten fields from one region of the epidermis provide a sufficiently precise estimate of mean cell size over the whole epidermis. The differences actually encountered in the experiment were many times greater than the fiducial limits of the means. Therefore, the final routine adopted for estimates of the number of cells per mm.^{2} was to sample from each leaf an area corresponding to *e* in Fig. 3 and to find the mean of ten random counts of cell number, excluding stomata, in this area.

(iii) *Total number of epidermal cells.* Since there is no significant variation in cell size from place to place on a fully expanded leaf, except at the extreme tip and edges, the product of area and the number of cells per unit area is an estimate of the total number of cells in the epidermis. Such an estimate is, of course, liable to errors; but the fiducial limits of mean cell counts are not high, and the errors are in any case separated out in the analysis of variance. The data may, therefore, be used with confidence. A significant change in the total number of epidermal cells might be due to a change in the number of cell divisions, or to a change in the size of the leaf primordium; it is not possible to distinguish in these data which of these two causes is the more important.

(iv) *Leaf shape.* The technique for measuring leaf shape will be discussed in the third contribution to this series.

4. SUMMARY OF DATA

The variables to be considered are: (i) three genotypes of *Ipomoea*, (ii) growing under two conditions of water supply (dry and wet), (iii) subjected to four operational treatments (control, A, B, C). There were ten plants under each of these twenty-four conditions. For each of the twenty-four conditions there are data for the area, cell size, cell number, and shape of each leaf at each node. Clearly the data cannot be published in full. The present section contains a summary of the data sufficient for a discussion of their bearing on the subject of this paper; more data will be found in the Appendix.

Morphology of leaves on control plants

The changes in leaf morphology from node to node on control plants are summarized in Figs. 4–12. All the measurements entered on the graphs are for leaves which were fully expanded. The circles represent data from house 1 (dry) and the crosses represent data from house 2 (wet). At this stage attention is drawn to the following features of the graphs; other features are discussed later.

Leaf area (Figs. 4–6). In strains x and z the second leaf had the greatest area and thereafter there was a steady fall in area from leaf to leaf up to about the eighth or ninth node. The first leaf was significantly smaller than the second. In strain y there was the same relation between leaf area and node of insertion, except that the second leaf was no larger than the first leaf. For all three strains, leaf areas under dry conditions were strikingly less than leaf areas under wet conditions. The analysis of variance merely confirms these observations (Table 2). In strain x there was a significant rise in leaf area between the sixth and the tenth leaves under dry conditions; strain z showed a similar rise, but it is significant only at the 10% level. These rises coincided with the period following heavy watering of the desiccated plants, after the sixth leaf had begun to enlarge (mentioned on p. 180).

Table 2. *Degrees of freedom* (D.F.), *variance ratio* (F), *and significance at the* 1 % *level* (S) *for leaf area of three strains of* Ipomoea *grown in dry and wet conditions*

Cause of variance	Strain x			Strain y			Strain z		
	D.F.	F	S	D.F.	F	S	D.F.	F	S
Node of insertion	12	13·54	S	14	7·21	S	11	10·34	S
House (wet or dry)	1	63·52	S	1	16·23	S	1	93·75	S
Interaction	12	1·49	—	14	1·87	—	11	4·98	S
Error	148	—	—	99	—	—	103	—	—

Number of cells per mm.^{2} (Figs. 7–9). All three strains showed a marked rise in the mean number of epidermal cells per mm.^{2} from leaf to leaf over the first few nodes. In strain *z* this rise continued up to the eleventh node. In strains *x* and *y* there was no significant increase in the number of cells per mm.^{2} after the fifth or sixth leaf. The unexpected and important feature of these data is that there is no striking difference in cell size between the leaves grown under dry conditions and those grown under wet conditions, notwithstanding the very highly significant differences in leaf area. This indication from the graphs is confirmed by the analysis (Table 3).

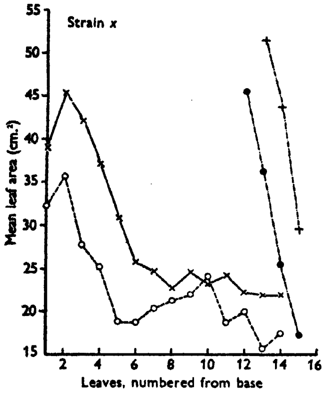

Fig. 4.

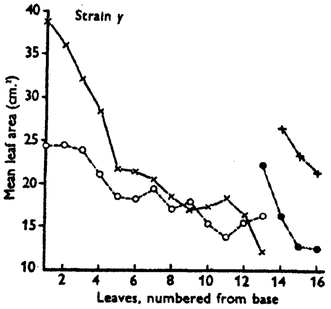

Fig. 5.

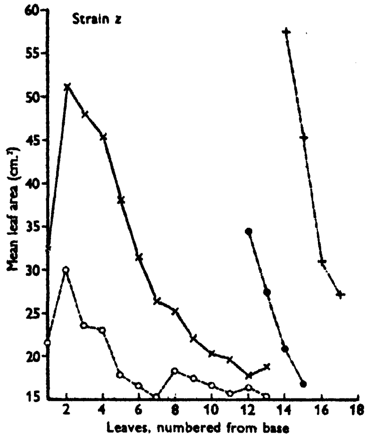

Fig. 6.

Figs. 4–6. Mean leaf area on control plants, plotted against position of insertion on the shoot; × ——— ×, wet conditions; ○ — — — ○, dry conditions. Mean leaf area of upper leaves on plants after treatment A; +·—·—·+, wet conditions; ●·—·—·●, dry conditions.

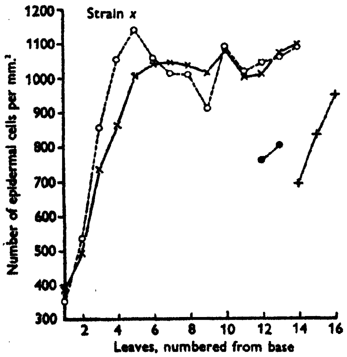

Fig. 7.

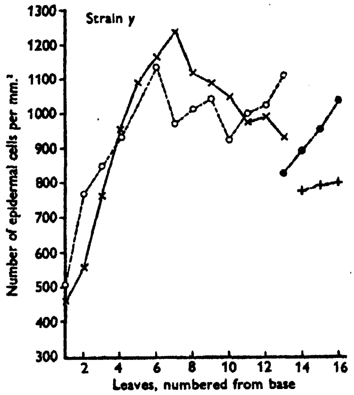

Fig. 8.

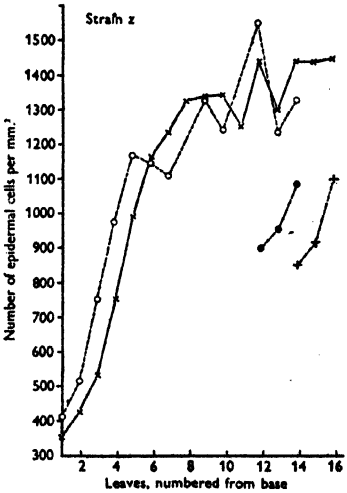

Fig. 9.

Figs. 7–9. Number of epidermal cells per mm.^{2} on leaves of control plants, plotted against position of insertion on the shoot; × ——— ×, wet conditions; ○ — — — ○, dry conditions. Number of epidermal cells per mm.^{2} on upper leaves of plants after treatment A; +·—·—·+, wet conditions; ●·—·—·●, dry conditions.

Total number of cells in epidermis (Figs. 10–12). In all three strains the total number of cells in the adaxial epidermis increased from the first to the fourth or fifth leaf; thereafter there was a gradual decline in cell number from leaf to leaf. There was an evident difference between the cell numbers, in leaves at corresponding nodes, grown in dry and wet conditions; and it is clear that in these varieties of *Ipomoea* the effect of water supply on leaf area operates through cell number and not through cell size. Under dry conditions strains *x* and *z* showed a significant rise of cell number in one or two successive leaves from the eighth leaf; these rises corresponded to the heavy watering of the desiccated plants after the sixth leaf had begun to enlarge. They are accompanied by increases in area, but the increases in area reached significance at an earlier leaf than the increases in cell number. The analysis of variance is summarized in Table 4.

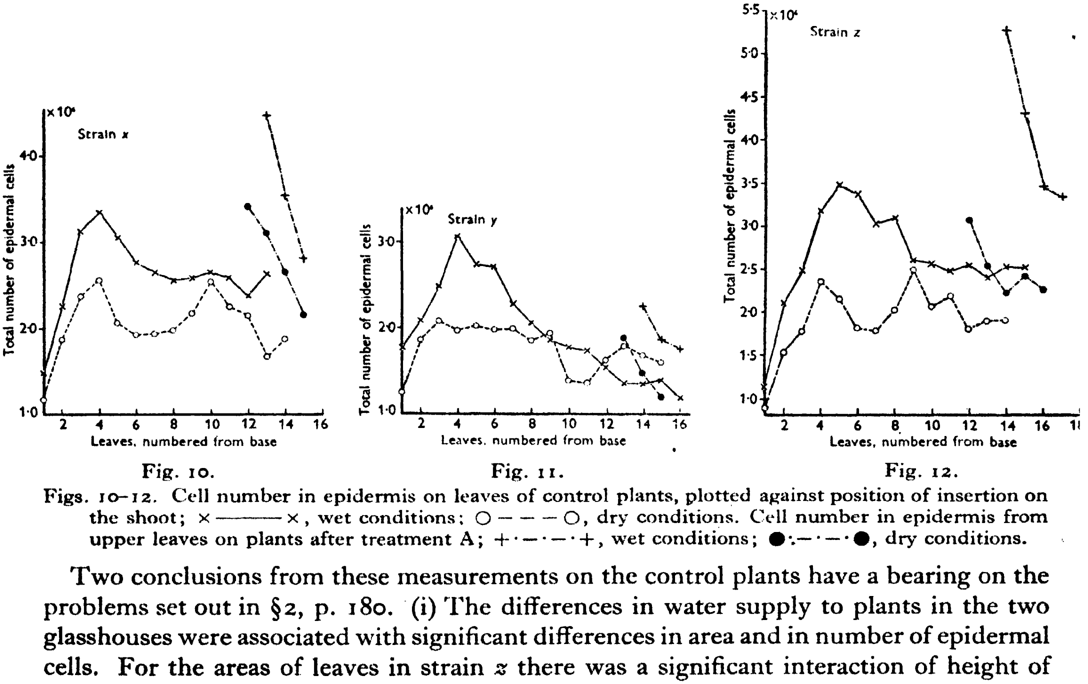

Fig. 10. Fig. 11. Fig. 12.

Figs. 10–12. Cell number in epidermis on leaves of control plants, plotted against position of insertion on the shoot; ×———×, wet conditions; ○ — — — ○, dry conditions. Cell number in epidermis from upper leaves on plants after treatment A; +·—·—·+, wet conditions; ●·—·—·●, dry conditions.

Two conclusions from these measurements on the control plants have a bearing on the problems set out in §2, p. 180. (i) The differences in water supply to plants in the two glasshouses were associated with significant differences in area and in number of epidermal cells. For the areas of leaves in strain *z* there was a significant interaction of height of insertion and water supply. But epidermal cell size, although it decreased significantly with node of insertion, was not significantly affected by water supply. This is an unexpected result, especially in view of the fact that in other plants, and under other conditions, the cell size of leaves is sensitive to water supply. (ii) Beside the familiar gradient of decreasing cell size from leaf to leaf, which is significant in all three strains, there is also a gradient from leaf to leaf in total number of cells and in leaf area.

Morphology of leaves from treated plants

Treatment A (Fig. 2). Attention is directed again to Figs. 4–12. The circles and crosses joined by alternate dots and dashes represent the mean dimensions of leaves $(n+1)$ $(n+2)$, $(n+3)$, etc., on plants which had received treatment A. These leaves developed with no older leaves below them. The following features are noteworthy:

(i) In all three strains the area of leaf $(n+1)$ was considerably larger than the area of the fully expanded leaf $(n+1)$ in the control plants. The effect is significant in both wet and dry conditions, and it is more marked in dry conditions.

(ii) There was a difference in the degree of response to treatment among the strains: strain y responded less than strains x and z.

Table 3. *Degrees of freedom* (D.F.), *variance ratio* (F), *and significance at the* 1 % *level* (S) *for cells per mm.*^{2} *in the adaxial epidermis of three strains of* Ipomoea *grown in dry and wet conditions.*

Owing to a lack of symmetry in the data from the two houses, the interaction variance cannot be interpreted with confidence, and is omitted; but there was no significant interaction when symmetrical sets of data from the two houses were analysed.

Cause of variance	Strain x			Strain y			Strain z		
	D.F.	F	S	D.F.	F	S	D.F.	F	S
Node of insertion	11	5·79	S	14	17·21	S	11	83·68	S
House (wet or dry)	1	0·20	—	1	0·03	—	1	3·80	—
Error	152	—	—	104	—	—	103	—	—

Table 4. *Degrees of freedom* (D.F.), *variance ratio* (F), *and significance at the* 1 % *level* (S) *for total number of cells in the adaxial epidermis of three strains of* Ipomoea *grown in dry and wet conditions.*

Owing to a lack of symmetry in the data from the two houses, the interaction variance cannot be interpreted with confidence, and is omitted

Cause of variance	Strain x			Strain y			Strain x		
	D.F.	F	S	D.F.	F	S	D.F.	F	S
Node of insertion	12	9·34	S	14	5·45	S	11	10·68	S
House (wet or dry)	1	72·2	S	1	31·7	S	1	69·6	S
Error*	206	—	—	175	—	—	151	—	—

* Since cell number does not change after a leaf is about 3 cm. long, fewer leaves had to be rejected from this analysis on account of their immaturity; this accounts for the greater number of degrees of freedom in Table 4 compared with Table 3.

(iii) The curves for area of leaves $(n+1)$, $(n+2)$, $(n+3)$, etc., fall very steeply in all three varieties, much more steeply than the curves of area of the corresponding control leaves in this region of the shoot. As a consequence, leaf $(n+4)$ in all varieties, although it had only three young leaves beneath it, was not significantly larger than the control leaf, which had about thirteen leaves beneath it. This is not a result which would be expected from the hypothesis that the sole effect of lower leaves is related to competition for water.

(iv) The cell size of leaves $(n+1)$, $(n+2)$, $(n+3)$, etc., is significantly greater if the leaves below have been cut off; but the increase in cell size is not as great as would be expected on the hypothesis that these leaves have available the *total* water supply from the roots; and from leaf $(n+1)$ to $(n+3)$ there is an unexpectedly rapid drop in cell size. Furthermore, the response of cell size in leaf $(n+1)$ is about the same whether conditions are wet or dry.

(v) The removal of leaves 1 to n had a strikingly significant effect on total cell number in the epidermis of leaves $(n+1)$, $(n+2)$, $(n+3)$, etc., except in strain y, where the effect was small. Since leaf $(n+1)$ was on an average about 0·8 cm. long when leaves 1 to n were removed, this indicates that cell division can be stimulated at a relatively late stage of leaf production. The data for total cell number in leaves $(n+1)$, $(n+2)$, $(n+3)$, etc., in common with the data for leaf area and cell size, show a very rapid recovery to the values of the controls; for instance, in strain *x*, leaf $(n+4)$ from treated plants, with three leaves below it, has about the same number of cells as leaf $(n+4)$ from control plants, with thirteen leaves below it. In all three strains the response of cell number to treatment was greater under wet than under dry conditions.

Treatments B and C (Fig. 2). These treatments were designed to test the simple hypothesis that the lower leaves affect the morphology of the upper leaves by virtue of their competitive effect on the water supply. The results are set out in Table 5. The predictions made in §2 (p. 180) are now considered in the light of Table 5. More complete data are given in the Appendix (p. 195).

Table 5. *Response of area, cell size, and cell number in leaf* $(n+1)$ *from three strains of* Ipomoea *to treatments A, B and C*

Response of leaf $(n+1) = R$, mean difference $= D$, probability of significance $= P$. Responses in brackets are significant at the 5 % level only. The analysis does not distinguish between responses under wet and dry conditions. Data in Appendix III.

Treatment	Strain *x*			Strain *y*			Strain *z*		
	Area (cm.^{2})	Cells/Area	Cell no.	Area (cm.^{2})	Cells/Area	Cell no.	Area (cm.^{2})	Cells/Area	Cell no.
A. *R*	More	Less	More	More	Less	(More)	More	Less	More
D	29·7	328	1·7	8·4	259	0·52	28·7	511	1·68
P	0·001	0·001	0·001	0·01	0·01	<0·05	0·001	0·001	0·01
B. *R*	None	None	None	None	None	None	None	(Less)	None
D	—	—	—	—	—	—	—	184	—
P	0·4	0·4	0·2	0·4	0·8	0·2	0·1	<0·05	0·5
C. *R*	More	Less	(More)	More	Less	More	None	Less	None
D	6·8	127	0·4	10·3	194	0·68	—	276	—
P	0·001	<0·01	0·05	0·001	0·04	0·03	0·6	0·03	0·6

In treatment B all but the uppermost leaves $(n-2)$, $(n-1)$, and *n*, were removed from the shoot (Fig. 2B). Therefore the development of leaves $(n+1)$, $(n+2)$, etc., took place free from competition from the adult leaves below, but not free from the effects of the three young leaves immediately below. If the morphology of upper leaves is affected principally by the competition for water from leaves below, then the response to treatment B should be similar to (though perhaps less than) the response to treatment A. Table 5 shows that this is not the case. Except for one set of observations (cell size in strain *z*, which is significantly different at the 5 % level), the morphology of leaves after treatment B is the same as that of leaves on control plants.

In treatment C only the three uppermost leaves $(n-2)$, $(n-1)$, and *n*, were removed from the shoot (Fig. 2C). Therefore the development of leaves $(n+1)$, $(n+2)$, etc., took place in the presence of all adult leaves below, and free only from the influence of the three young leaves immediately below. If the morphology of upper leaves is affected principally by the competition for water from leaves below, then there should be no significant response to treatment C. Table 5 shows that this, too, is not the case: there were significant responses to treatment C, except for two sets of observations with strain *z*, which showed no response. Apart from this inconsistency in strain *z*, treatment C produced the same sort of responses as treatment A; indeed in one strain (strain *y*) even the mean differences between observations on leaf $(n+1)$ from treated and control plants are no less for treatment C than for treatment A. The general conclusion to be drawn from Table 5 is that the area, cell size, and cell number of a developing leaf are greatly modified by the influence of the immature leaves immediately below, but are not significantly modified by the influence of as many as 8–10 adult leaves below.

An analysis of the response of the lowest leaf $(n-2)$ left on the plant in treatment B reveals another relevant point. This leaf had already enlarged to about half its normal size when the leaves below it were cut off, yet it responded significantly to the operation. The response was, as would be expected, confined to cell size and area; there was no response in total cell number. The evidence is summarized in Table 6. The result indicates that the final cell size and consequently leaf area, can still be influenced even when the leaf has reached half its normal adult size, while cell number can no longer be influenced at this stage.

Table 6. *Response of leaf* $(n-2)$ *to treatment B* (see Fig. 2)

Strain		Area (cm.^{2})	$\frac{\text{Cells}}{\text{Area}}$	Cell no.
x	Response Mean difference *P*	More 16·2 0·001	Less 336 0·001	None — 0·1
y	Response Mean difference *P*	None — 0·08	Less 238 0·001	None — 0·6
z	Response Mean difference *P*	More 15·9 0·01	Less 586 0·001	None — 0·4

When treatment C was applied, the topmost leaf left on the shoot, $(n-3)$, was not fully grown. An analysis of its response to the treatment gave inconclusive results. There was no response of leaf $(n-3)$ in strains *x* or *y*, and there was a slight response, significant at the 5% level, in strain *z*. The data do not merit further discussion at this stage.

5. DISCUSSION

The data presented in this paper are relevant to several problems of leaf development; at this stage discussion will be confined to two of these problems, namely: (i) to what extent are leaf area, cell size, and cell number influenced by dry and wet conditions of cultivation? and (ii) to what extent is the decrease in cell size from leaf up the shoot due to competition for water among the leaves? The evidence for answering these questions is as follows:

When groups of *Ipomoea* plants grown under dry conditions were compared with groups grown under wet conditions (conditions of light and temperature being similar for the two groups) it was found that corresponding leaves between groups differed significantly in area and in cell number, but not in cell size. In both groups of plants there was a decrease in cell size from leaf to leaf. This gradient was not significantly greater under dry conditions than under wet conditions, and in two of the three strains it was not maintained after the sixth to eighth leaf. This evidence indicates that, within the limits of the experimental conditions, differences in water supply affect leaf area and cell number in the epidermis, but do not affect cell size; also that the decrease in cell size from lower to upper leaves is independent of the wet and dry conditions of cultivation.

The four treatments illustrated in Fig. 2 support and extend this conclusion. If all leaves on the shoot (1 to n) except those in the terminal bud are cut off, the next leaf to develop $(n+1)$ has a larger area, larger cells, and more cells, than the corresponding leaf on control plants. If this response were due simply to the release of water formerly monopolized by the lower leaves, then treatment B (Fig. 2) would influence leaf $(n+1)$ in the same way, if not to the same degree, as treatment A; and treatment C (Fig. 2) would influence leaf $(n+1)$ in the same way, if not to the same degree, as no treatment at all. The results of the treatments, summarized in Table 5, are quite contrary to these predictions; the data clearly show that it is not the bulk of adult leaves, but the three or four immature leaves immediately below a developing leaf, which affect the area, cell size, and cell number of the developing leaf. The effects are highly significant in both wet and dry environments. Furthermore, in successive leaves $(n+1)$, $(n+2)$, $(n+3)$, ..., there is a rapid recovery from the effects of treatments A, B and C; so that in most cases leaf $(n+4)$ in treated plants, which has only three young leaves below it on the shoot, does not differ significantly from leaf $(n+4)$ in untreated plants, which has about thirteen leaves below it.

This evidence is plainly inconsistent with the generally accepted hypothesis that upper leaves have smaller cells (and other xeromorphic characters) *because* lower leaves deprive the upper leaves of water. The evidence is, however, consistent with another hypothesis: that area, cell size, and cell number in a growing leaf are influenced either by substances which diffuse upwards from immature leaves below, or alternatively, by a deficit of substances which are monopolized by the immature leaves below. This proposed hypothesis accounts for the differences between the responses to treatments B and C, and for the fact that the responses to treatment do not persist after the first two or three leaves (i.e. leaf $(n+4)$ in treated plants is almost a 'normal' leaf). The proposed hypothesis is consistent also with the fact that a gradient of decreasing cell size exists from leaf to leaf among the first few leaves on the shoot: for there is an increase in the *tempo* of leaf production, resulting over the first few nodes in an increasingly large number of immature leaves below any developing leaf. In strain x, for instance, the data on leaf dimensions reveal the following information (Table 7): there is a high correlation between the number of immature leaves below an unfolding leaf, and the cells per mm.^{2} in the unfolding leaf when it is fully grown. In treatment C it was shown that even when a leaf is half grown it will still respond to the removal of leaves immediately below it, provided they are not all quite mature. The response is confined to cell size and (consequentially) area: there is no change in cell number. There was no evidence that treatments A, B or C brought about carbohydrate deficiency in the plants.

It cannot be assumed that the conclusions drawn from this experiment are applicable to other plants under other conditions; there were indeed differences in response to treatments even among the three strains of *Ipomoea* used. However, the conclusions proposed in this paper do raise general issues of some importance in physiological anatomy and do imply that some current opinions should be revised. Therefore it is necessary to inquire whether the data of other workers are consistent with the conclusions proposed here. There are already in the literature several facts which throw doubt on the commonly accepted hypothesis of the relation between water supply and leaf structure. Even the data of Alexandrov *et al.* (1921), an example of which is reproduced in Fig. 1, do not fully support their authors' conclusion: for if cell size in developing leaves is determined by competition for water among the leaves below, then the curve of stomatal frequency should continue to rise as more leaves enter the competition. This is not in fact the case. Apart from spurious rises due to the inclusion of immature leaves in the samples, the stomatal frequency becomes stable in Alexandrov's data at 200–300 per mm.^{2} from the twentieth to the seventieth leaf. Data of Zalenski (1904), Rippel (1919) and others support the view that stomatal frequencies do not rise indefinitely with level of insertion on the shoot. Yapp (1912) recorded several observations which are inconsistent with the current hypothesis. He found, for instance, that in successive leaves of a non-flowering shoot of *Spiraea Ulmaria* there was not a gradient of increasing stomatal frequency from leaf to leaf, but an increase to a maximum in the summer, followed by a decrease in autumn.

Table 7. *The state of development of leaves below a young leaf, when the young leaf is* 0·8 *cm. long; together with the number of cells per mm.*^{2} *when the leaf is fully grown. Strain x*

No. of young leaf	$\frac{\text{Cells}}{\text{mm.}^2}$	Adult leaves below	Immature leaves below
1st	385	0	0
2nd	506	0	1
3rd	759	0	2
4th	864	1	2
5th	1027	1	3
6th	1082	1	4
7th	1132	2	4
8th	1171	3	4
9th	1103	4	4
10th	1098	5	4
11th	1005	5	5
12th	1008	6	5

He found, too, that there was a marked difference in stomatal frequency between basal and distal pinnae of the same leaf; in one particular plant the first leaf on the shoot was more xeromorphic in this and other respects than the basal (but not the distal) pinna on the sixth leaf. In studying hairiness of leaves, which is highly correlated with cell size, Yapp found less variation between sheltered and exposed habitats than he expected; and his observations led him to the suggestion that the glabrous leaves sometimes induced by removing lower leaves on a shoot, and the glabrous leaves found occasionally above injured parts of the stem, may be caused by the injuries themselves, and not by the consequential disturbances in water supply. These scattered facts are consistent with the conclusions advanced in the present paper.

There is an apparent discrepancy between the independence of cell size and water supply reported in this paper and the close dependence of cell size on water supply reported by some other workers. Many of the data which support the view that dry conditions produce smaller cells are concerned with sun and shade leaves, where not only moisture, but light also, will have affected cell size. However, both Rippel (1919) and Tumanov (1927) have observed that plants grown in dry soils have small cells. In Rippel's experiments the 'dry' plants were very dwarfed indeed, and in Tumanov's experiments the 'dry' plants were repeatedly wilted. Cell size may well be affected by such extreme conditions of drought as these, even though it is not affected by the less extreme conditions provided in the present author's experiments. It is noteworthy that even the 'Trockenzwerge' of Rippel did not have consistently smaller cells than normal plants, and the dwarfness of their leaves was due primarily to their having fewer, and not smaller, cells, than normal plants.

Out of the conclusions presented in this paper, three important questions arise, which cannot be answered from the data. They are: (i) To what extent is leaf structure determined by the external environment at the time the leaf unfolds? Both Zalenski and Yapp considered that the structure of a leaf is affected more by its position on the shoot than by the time of year it appears; but there are no critical data which enable these two variables to be separated. (ii) What is the relation between the structure and the physical properties of a leaf (e.g. between cell size and osmotic pressure, suction force, elasticity of cell walls, etc.)? (iii) If the structure of a growing leaf is influenced by the three or four immature leaves below it, what is the nature of this influence? Is the influence due to substances diffusing upwards from the immature leaves, as data of Goodwin (1937) and Harder & von Witsch (1940) might suggest? Or is it due to interception, by the immature leaves, of substances beneficial to leaf growth?

Experiments designed to answer these questions are in progress in the author's laboratory.

6. SUMMARY

1. In this paper the author examines the question of the relation between cell size in leaves and their level of insertion on the shoot.

2. Experiments with *Ipomoea* plants were designed to test the current hypothesis that the gradient of decreasing cell size from lower to upper leaves on a shoot is due to competition for water among the leaves. Four treatments were applied, illustrated by the following example:

(i) Control. Single shoots grown to 16-leaf stage.
(ii) A. Leaves 1–10 removed; response studied in leaves 11–16.
(iii) B. Leaves 1–7 removed; leaves 8–10 left on; response studied in leaves 11–16.
(iv) C. Leaves 8–10 removed; leaves 1–7 left on; response studied in leaves 11–16.

Three strains of *Ipomoea* were used. Parallel experiments were conducted under wet and dry conditions. Observations were made on leaf growth, leaf area, and the size and number of adaxial epidermal cells.

3. The principal conclusions from analyses of the data are:

(i) There are significant gradients in area, cell size, and cell number from leaf to leaf up the shoot.

(ii) Leaf area and cell number are significantly affected by water supply, but cell size is not affected.

(iii) In respect of area, cell size, and cell number, leaves 11, 12, ..., respond to treatment B in the same way as to the controls (i.e. to no treatment at all); and they respond to treatment C in the same way as to treatment A. Therefore leaves 11, 12, ..., do not respond to the removal of seven adult leaves below them, but they do respond to the removal of three young leaves below them.

4. On this and other evidence the view is put forward that the gradients of leaf area, cell size and cell number between successive leaves on a shoot are not the result of competition for water among adult leaves on the shoot, but are related to the influence of immature leaves upon still younger leaves developing above them. This influence may operate either through the diffusion upwards, from immature leaves, of substances which inhibit cell division and expansion in still younger leaves; or alternatively, it may operate through the interception, by immature leaves, of substances which promote cell division and expansion in still younger leaves.

5. According to this view, the relatively xeromorphic characters of upper leaves compared with lower leaves, in so far as they are dependent on cell size, are attributable principally to the influence of immature leaves immediately below the apex, affecting the morphology of leaves developing at the apex. Any disturbance of water economy which may result from the removal of all adult leaves from a shoot, does not, in the strains of *Ipomoea* studied, influence the morphology of leaves developing at the apex.

6. The consequences of this view are being examined by a further series of experiments.

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REFERENCES

ALEXANDROV, W., ALEXANDROV, O. & TIMOFEEV, A. (1921). The water supply of leaves and their structure. *Sci. pap. appl. sect. Tiflis bot. Gdn*, **2**, 85. (Russian.) See also Maximov (1929), p. 342, and Walter (1926), p. 70.

ASHBY, E. (1948). Studies in the morphogenesis of leaves. I. An essay on leaf shape. *New Phytol.* **47**, 153–76.

FISHER, R. A. & YATES, F. (1943). *Statistical Tables.* Edinburgh.

GOODWIN, R. H. (1937). The role of auxin in leaf development in *Solidago*. *Amer. J. Bot.* **24**, 43.

HARDER, R. & VON WITSCH, H. (1940). Über den Einfluss der Tageslänge auf den Habitus, besonders der Blattsukkulenz und den Wasserhaushalt von *Kalanchoë Blossfeldiana*. *Jb. wiss. Bot.* **89**, 354.

IMAI, Y. (1930). A genetic monograph on the leaf form of *Pharbitis Nil*. *Z. indukt. Abstamm- u. VererbLehre*, **55**, 1.

MAXIMOV, N. A. (1929). *The Plant in Relation to Water*, ed. R. A. Yapp. London.

RIPPEL, A. (1919). Der Einfluss der Bodentrockenheit auf den anatomischen Bau der Pflanzen. *Beih. z. bot. Ges.* **36**, 187.

TUMANOV, J. J. (1927). Ungenügende Wasserversorgung und das Welken der Pflanzen, usw. *Planta*, **3**, 391.

WALTER, H. (1926). *Die Anpassungen der Pflanzen an Wassermangel.* Naturwissenschaft u. Landwirtschaft, H. 9. München.

YAPP, R. H. (1912). *Spiraea Ulmaria* and its bearing on the problem of xeromorphy in marsh plants. *Ann. Bot., Lond.*, **26**, 815.

ZALENSKI, V. (1904). Materials for the study of the quantitative anatomy of different leaves on the same plant. *Mém. Poly. Inst. Kiev*, **4**, 1 (Russian).

APPENDICES

I. *Correction for number of incomplete cells in microscopic fields* (§(ii), p. 182)

When the outlines of cells are irregular it is very laborious to count the number of incomplete cells in a microscopic field. Therefore the author calculated a regression from which a correction equal to half the number of incomplete cells could be estimated from the number of complete cells in a field. He is indebted to Prof. M. S. Bartlett, of the University of Manchester, for suggesting a suitable formula for this regression, derived as follows:

Let the area of the square field $= l^2$ and the mean area of a cell $= a$. Then if there are n whole cells and m incomplete cells in the field, and if n is large,

$$l^2 = (n + \tfrac{1}{2}m)\, a.$$

Let $h =$ the average length of a chord of a cell intersected by the edge of the field. Then

$$mh = 4l \quad \text{and} \quad l^2 = m^2h^2/16$$

If $h^2/a = l/c$ then

$$m^2 - 8mc = 16nc,$$

$$m^2 - 8mc + 16c^2 = 16nc + 16c^2,$$

$$(m - 4c)^2 = 16c(n + c),$$

$$m - 4c = 4\sqrt{[c(n + c)]},$$

and

$$\tfrac{1}{2}m = 2\{\sqrt{[c(n + c)]} + c\}.$$

This formula will not hold exactly when n is not large, especially as it neglects the effects of the corners of the square field; but it suggests that a linear regression of $\frac{1}{2}m$ on $\sqrt{n}$ should be found to be a sufficiently accurate relationship. The goodness of fit of the regression is illustrated by Fig. 13.

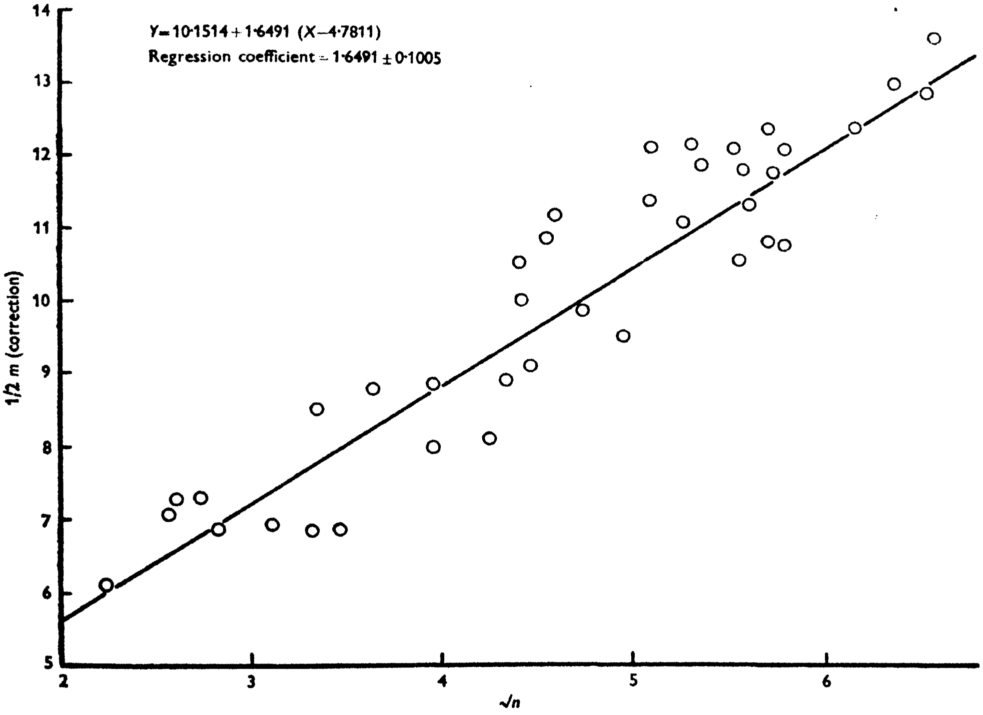

Fig. 13. Regression of half the number of incomplete cells in a microscopic field on the square root of the number of complete cells in the field.

II. *Control plants: mean values of leaf area, cell size, and cell number, used in analysis of data*

Leaf			1	2	3	4	5	6	7	8
Area (cm.^{2})	*x*	Dry	32·2	35·7	28·4	25·0	18·1	18·0	19·8	20·9
		Wet	39·0	46·1	43·6	39·2	31·2	26·1	24·2	22·1
	y	Dry	24·5	23·5	23·4	20·4	18·5	19·2	19·2	17·8
		Wet	37·4	34·2	31·0	28·5	22·3	21·2	20·5	18·3
	z	Dry	21·6	30·0	23·6	23·2	17·8	16·2	15·0	18·2
		Wet	32·6	51·3	50·1	47·7	40·4	33·4	27·2	25·3
$\frac{\text{Cells}}{\text{mm.}^2}$	*x*	Dry	365	525	836	1044	1115	1030	1019	1007
		Wet	385	506	759	864	1027	1082	1132	1171
	y	Dry	503	751	854	972	1054	1082	1026	909
		Wet	438	548	779	1014	1125	1213	1241	1112
	z	Dry	408	517	754	1009	1221	1202	1194	1332
		Wet	347	430	532	726	977	1168	1241	1292
Cell no. × 10^{-6}	*x*	Dry	1·17	1·87	2·38	2·55	2·06	1·91	1·92	1·98
		Wet	1·48	2·27	3·14	3·35	3·06	2·77	2·67	2·57
	y	Dry	1·24	1·87	2·08	1·99	2·01	1·98	1·99	1·84
		Wet	1·80	2·18	2·51	3·00	2·71	2·63	2·31	2·07
	z	Dry	0·88	1·58	1·78	2·36	2·17	1·81	1·77	2·03
		Wet	1·12	2·10	2·49	3·19	3·48	3·38	3·01	3·10

Leaf			9	10	11	12	13	14	15
Area (cm.^{2})	*x*	Dry	24·3	24·0	21·8	20·3	15·8	—	—
		Wet	23·1	20·8	24·1	22·4	22·5	—	—
	y	Dry	20·4	16·8	14·0	16·3	16·4	14·5	15·4
		Wet	17·3	17·3	18·1	16·3	12·9	13·4	14·0
	z	Dry	17·5	16·7	16·0	16·4	—	—	—
		Wet	22·7	20·4	19·9	17·9	—	—	—
$\frac{\text{Cells}}{\text{mm.}^2}$	*x*	Dry	911	1082	1019	1042	—	—	—
		Wet	1103	1098	1005	1008	—	—	—
	y	Dry	1056	883	1009	977	1244	1161	1041
		Wet	1087	1045	976	976	902	998	997
	z	Dry	1485	1237	1270	1576	—	—	—
		Wet	1315	1365	1249	1440	—	—	—
Cell no. × 10^{-6}	*x*	Dry	2·19	2·56	2·24	2·14	1·68	—	—
		Wet	2·59	2·41	2·60	2·24	2·65	—	—
	y	Dry	1·98	1·40	1·36	1·55	1·80	1·69	1·60
		Wet	1·84	1·76	1·72	1·54	1·35	1·34	1·39
	z	Dry	2·50	2·07	2·28	—	—	—	—
		Wet	2·64	2·57	2·48	—	—	—	—

III. *Differences between leaf* $(n+1)$ *on treated plants and the mean for the corresponding leaf on control plants*

Treatment ...	A			B			C		
Strain	*x*	*y*	*z*	*x*	*y*	*z*	*x*	*y*	*z*
				Area (cm.^{2})					
Dry	38·4	3·9	29·9	−0·4	−4·0	1·0	8·5	7·2	4·2
	28·9	4·8	16·2	4·8	2·4	—	7·0	6·8	—
	24·7	8·5	20·7	1·9	−4·5	—	6·5	13·7	—
	11·0	—	8·8	—	—	—	—	—	—
Wet	6·4	9·1	33·1	−2·2	14·2	8·5	5·2	9·8	−0·8
	38·4	15·9	42·2	−4·8	1·7	2·3	—	14·1	12·3
	48·0	—	50·2	0·3	4·7	5·3	—	—	−6·7
	45·4	—	—	13·7	—	—	—	—	—
	26·2	—	—	—	—	—	—	—	—
				Cells per mm.^{2}					
Dry	307	197	321	44	−255	326	119	153	490
	305	327	384	120	475	—	−6	434	385
	289	463	696	−199	−97	—	27	173	—
	332	—	336	−184	—	—	184	—	—
	—	—	—	—	—	—	67	—	—
Wet	420	208	637	−210	121	314	152	56	335
	445	−2	671	−154	165	125	287	152	−147
	516	361	536	−30	−181	18	188	—	315
	224	—	—	65	−159	137	—	—	—
	114	—	—	162	35	—	—	—	—
				Total no. of cells $\times 10^{-6}$					
Dry	2·42	0·03	2·36	−0·28	−0·03	−0·45	0·86	0·39	−0·75
	1·48	0·14	0·57	0·25	−0·35	—	0·63	0·08	—
	1·46	0·14	0·27	0·79	−0·28	—	0·18	1·19	—
	0·08	—	0·25	0·44	—	—	—	—	—
Wet	1·43	1·21	1·98	1·98	0·77	0·08	0·23	0·85	2·23
	1·56	0·91	2·53	−0·78	0·54	0·15	0·12	0·93	1·62
	3·08	0·69	3·81	—	0·44	0·72	—	—	3·50
	2·22	—	—	—	0·61	0·11	—	—	—

STUDIES ON THE PHYTOSTATIC ACTION OF 2:4-DICHLOROPHENOXYACETIC ACID AND COUMARIN

THE REVERSIBILITY OF ROOT-GROWTH INHIBITIONS

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(With Plate 4 and 11 figures in the text)

INTRODUCTION

The successful application of the root-growth inhibiting propensities of plant-growth substances to the control of weeds of arable land has brought an academic and, at one time, remote botanical problem into the glaring light of present-day applied research. The extremely high sensitivity and differential nature of the response of plants to such synthetic growth substances as 2:4-dichlorophenoxyacetic acid and 4-chloro:2-methylphenoxyacetic acid have been very largely responsible for their practical success in the field. So striking is this action that these compounds are now referred to as selective 'phytocidal' agents and tend to be mentally relegated to the category of such purely destructive lethal agents as sulphuric acid, etc. Death, however, means irreversibility, and it is difficult to see how we can reconcile such a lethal action of growth substances on roots with their growth-stimulating properties in shoots. Thus, the same small external concentration of heteroauxin can bring about a large stimulation of the longitudinal growth of such an organ as an oat coleoptile and yet inhibit almost completely the longitudinal growth of the root. It has been suggested that this inhibition is a case of supra-optimal action (Boysen-Jensen, 1936), since many workers on both excised and intact roots claim to have shown that low concentrations of auxins of the order of 1 part in 10^{10} bring about an actual stimulation of growth in length of roots (Amlong, 1936; Fiedler, 1936; Thimann, 1936; Geiger-Huber & Burlet, 1936; Grace, 1937; Macht & Grumbein, 1937; Weiler, 1938; Borgström, 1939*b*). Implicit however in the term 'hormones', as applied to these substances, is the concept of control of the degree and nature of growth, as we see it exemplified in the vast majority of phenomena associated with auxin action (viz. tropisms, nastic movements, lateral bud inhibitions, rooting of cuttings, etc.). Clearly, then, this inhibition of longitudinal root growth may be regarded as an example of 'over-control' by excess hormone, a condition which should disappear as soon as the controlling agent has been removed, assuming, of course, that serious autolytic changes have not set in during the period of inhibition as a result of disturbances in the dynamic growth equilibrium. In other words, to adopt the phraseology of the biochemist, root-growth inhibition by auxins, if not maintained for too long a period, should be a reversible process.

In spite of its fundamental nature, this postulate has been the subject of remarkably little direct experimentation. In fact, the author has been able to trace only three references to incidental observations of the reversibility of action of heteroauxin on roots. Thus, Neilsen (1930) found that the inhibiting action of rhizopin (subsequently shown to be heteroauxin) on longitudinal root growth of lupin was completely reversible after inhibition periods of 1 hr. Again, Thimann & Lane (1938) showed similar recovery of root growth in oat after inhibitions of 24–48 hr. with 200 p.p.m. heteroauxin. These workers claim to have demonstrated a stimulation of longitudinal root growth on recovery from the inhibition. This was assumed to be due to the rapid reduction of auxin concentration in the root cells to a very low value falling within the stimulation range mentioned above. Finally, Delarge (1941) found that heteroauxin stimulated the production of numerous lateral root meristems in excised roots of maize. The further growth and elongation of these roots were inhibited by auxin, but slight growth in length was obtained on transference to a pure culture medium.

In the work to be described, therefore, this question of the reversibility of growth-substance action in inhibiting root growth has been subjected to more direct and intensive experimentation. In view of its immense practical importance in the field and the high stability of the molecule in unsterilized water culture media, 2:4-dichlorophenoxyacetic acid (2:4-D.) was chosen as the experimental growth substance. In addition, so much data on its inhibiting action under varying conditions had already been accumulated as to make it a convenient material for this work. Parallel experiments were also carried out on the plant metabolite coumarin, a substance already shown to have marked inhibiting action on root growth in small concentrations (Audus & Quastel, 1947*a*). Certain similarities in the actions of coumarin and auxins suggested investigation into the reversibility of its inhibition of longitudinal root growth.

PRELIMINARY EXPERIMENTS

The preliminary experiments were carried out on samples of 200–250 cress seedlings, germinated and grown on the surfaces of water culture medium in 400 ml. beakers. 200 ml. of dilute buffered water-culture medium (pH 6·8) of the same constitution as that employed in previous experiments (see Audus & Quastel, 1947*a*) were used, and the seeds supported at the surface on disks of bakelite perforated by 1 mm. holes. The disks themselves were held in position by glass tripods immersed in the medium. Roots grew easily through the holes and the seedlings remained upright, being supported by contact with neighbouring seedlings and the sides of the beaker. A number of such beakers were set up, one acting as a control and containing pure culture medium, and the remainder containing culture medium with an added concentration of 1 p.p.m. of 2·4-dichlorophenoxyacetic acid. Growth took place at laboratory temperature (approx. 20° C.) and in normal daylight of a south-facing window.

Observations on the growth of seedling samples were made by two main methods. The first method consisted of lifting out the bakelite disk with the seedlings *in situ*, removing as much surface water as possible from the roots and disk by means of filter-paper and weighing the whole to the nearest milligram. Having previously determined the combined weight of disk plus soaked seed, the gain in fresh weight of the seedlings over the interval could be quickly ascertained. After weighing, disk and seedlings were replaced in the culture medium for further growth. For investigating reversibility, the medium containing 2:4-D. was replaced by pure medium after set intervals, the seedlings and disk being thoroughly washed with distilled water during the transfer.

Fig. 1 shows results of a typical experiment of this type. The graphs represent the total growth of an individual sample of 200–250 seedlings, expressed as a total gain in weight in grams per 10 g. of soaked and fully swollen seeds. The vertical arrows mark the times at which the corresponding seedling samples were transferred to pure culture solution.

A comparison of the two control curves clearly illustrates the effect of 1 p.p.m. 2:4-D. on the normal growth of seedlings. Development is considerably delayed, and total growth reduced to about a seventh normal. Samples washed and transferred to normal culture, after 1, 2, 4 and 7 days, show intermediate growth curves, thus demonstrating clearly the reversibility of 2:4-D. inhibition. The curves suggest that growth rate starts to increase immediately after the removal of the inhibitor.

These curves, however, only give a very rough indication of the reversibility phenomena and are subject to considerable errors, due to varying amounts of water left adhering to the samples during weighing. In addition, the method fails to distinguish between effects on roots and effects on shoots, the former being much more marked than the latter. In the second type of experiment therefore no attempt was made to weigh whole samples of 200 or so seedlings. Instead, random samples of 10 seedlings were removed from each beaker from time to time, their roots cut off with a sharp scalpel, dried on filter-paper and weighed rapidly to the nearest 0·1 mg. Curves from a typical experiment are seen in Fig. 2, where a similar transfer procedure was adopted (after 2, 3, 5, 7 and 9 days). The curves will be seen to follow a course similar to those in Fig. 1, indicating a recovery from inhibition as soon as the hormone is removed. These curves, however, are much more subject to fluctuation than those of the preceding figure, due to the large sampling errors inherent in this method. They do, however, demonstrate the greater sensitivity of the root to 2:4-D. inhibition and suggest that, at least for the first four graphs of the series (inhibition for from 2 to 7 days), normal growth rate is eventually attained after washing.

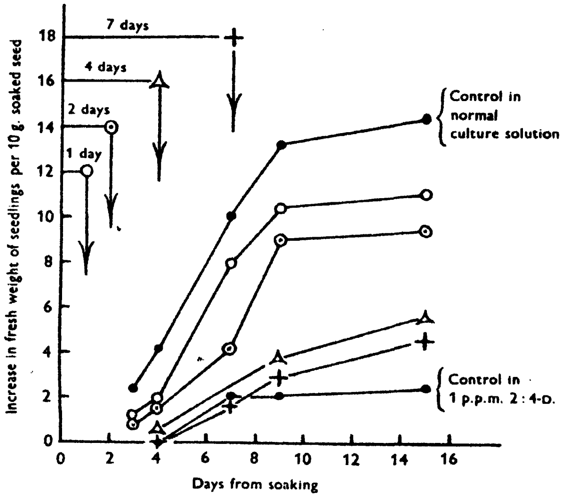


Fig. 1. Graphs of total growth of samples of cress seedlings showing the effect of varying periods of exposure to 1 p.p.m. 2:4-D. The vertical arrows mark the times of washing of the relevant samples.

Owing to the large errors inherent in this beaker technique, and in order to obtain more accurate information on the time and concentration relations of this inhibition and subsequent recovery, it was decided to employ a method whereby the growth in length of individual roots could be accurately studied during periods of inhibition and reversal. The final technique evolved is described in the following section.

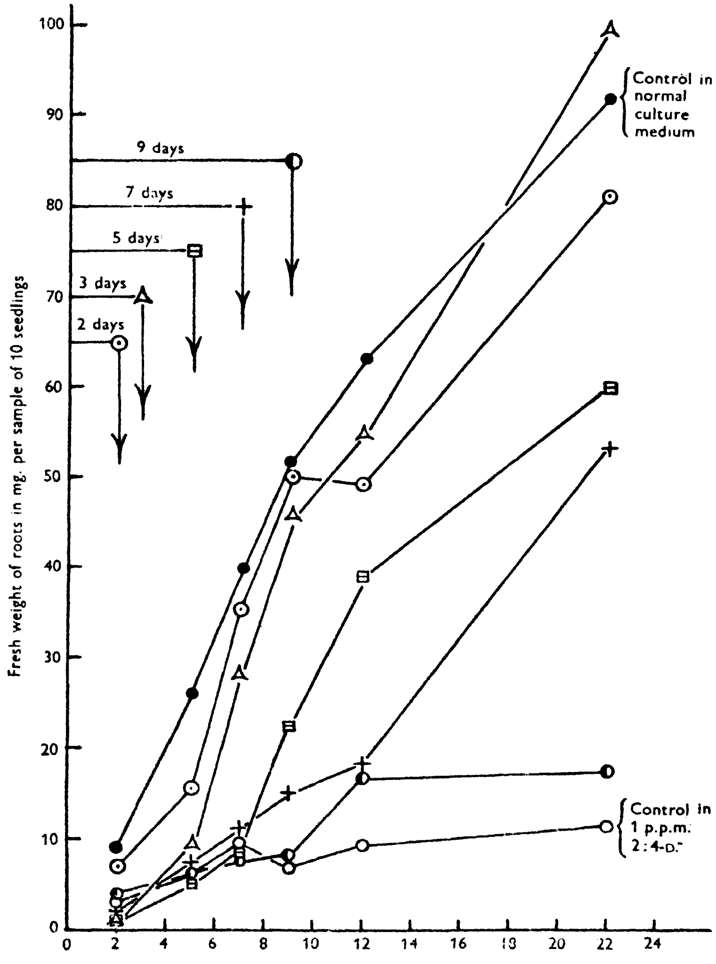


Fig. 2. Graphs of root growth of samples of cress seedlings showing the effect of varying periods of exposure to 1 p.p.m. 2:4-D. The arrows mark the times of washing of the relevant samples.

APPARATUS

The apparatus used in the main experiments is illustrated in Fig. 3. Except for two rubber bungs it is constructed entirely of glass. Tube *A* is of ½ in. internal diameter, the seedling studied being suspended just in contact with the surface of the culture medium, which fills the whole apparatus up to about ½ in. above the base of tube *A*. The method of suspension of the seed differed with the species used. In the case of cress, the soaked and sterilized seed was held in position at the bottom of glass tube *C* by a small filament of glass rod fused across the base. The radicle grew easily through one of the two holes thus produced and down into the narrower vertical tube *B*. In the case of pea, the soaked and surface-sterilized seed was supported on a small tripod of glass *T*, which allowed free growth of root down into tube *B*. By the application of a slight and steady suction to tube *C*, air could be drawn in at tube *D* and caused to pass up tube *E* in the form of bubbles. By careful choosing of the diameter of tubes *D* and *E*, the bubbles could be made to 'fit' tube *E* and, in their passage upwards into *A*, bring about a rapid circulation of culture fluid from the conical flask reservoir. By this simple means the root was kept constantly bathed in an aerated circulating buffered medium, thus maintaining conditions constant for periods of weeks. The air entering at *D* was taken from outside the laboratory to avoid gas contaminants such as ethylene, which is known to have such marked physiological effects, especially in relation to auxin action (see Borgström, 1939*a*). Temperature was maintained approximately constant at 20° C. and recorded on a thermograph. Seedlings were illuminated from the time of germination onwards by artificial light of a day length of 9 hr.

The growth of the root was measured at suitable time-intervals by means of a vertically travelling microscope with a vernier scale allowing of accurate measurements down to 0·05 mm. Measurements of growth of pea roots were simple, a datum point being marked on the tube *A* at the approximate position of emergence of the radicle, all lengths being determined from the datum point. With cress, however, difficulties were sometimes encountered, as a result of the base of the hypocotyl descending through the hole in tube *C*. In this case, therefore, the point of insertion of the uppermost root hairs was taken as a datum point. This was always clearly visible, as the root hairs soon became outlined by a deposit of calcium-phosphate particles present in the circulating culture medium. The time intervals over which growth was determined were relatively large and no attempt was made to measure small diurnal growth rhythms over short time intervals, since the main concern of the experiment was an investigation of the gross progress of growth as seen in the smooth curve of Fig. 4.

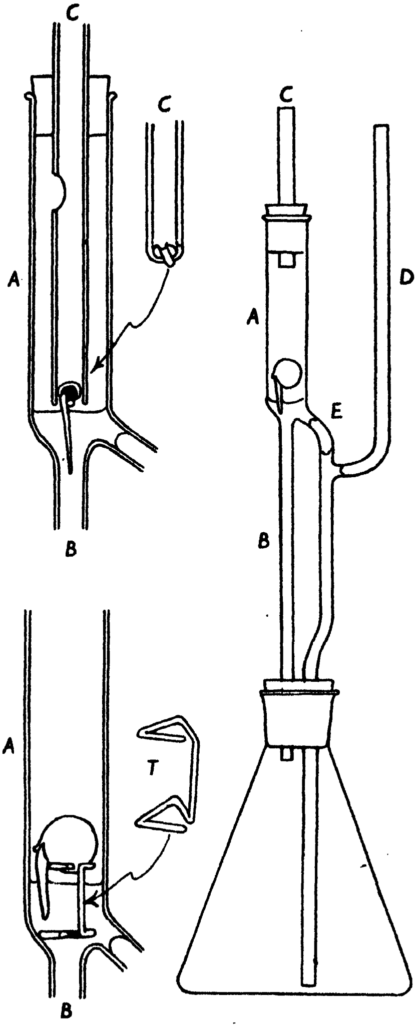


Fig. 3. Diagram of the apparatus used for experiments on the reversibility of growth inhibitions caused by 2:4-D. and coumarin. (For explanation see text.)

Experimental procedure consisted in germinating the seeds in pure culture medium and allowing the roots to grow down into the tube *B*. At a suitable time the reservoir was removed, refilled with culture medium containing a known concentration of inhibitor, and then replaced, and the fluid topped up to the correct level with the same culture medium. This whole procedure took no more than 2 min. to perform. Subsequent growth was followed in the circulating medium. After varying time intervals, the reservoir was again removed and washed with distilled water, together with the rest of the apparatus, care being taken not to disturb the position of the seedling during the process. The whole apparatus was then refilled with fresh culture fluid, circulation started and further growth determined.

NORMAL GROWTH CURVES

Typical progress curves of the normal growth of the main radicle of cress and pea, under the growth conditions described, are shown in Fig. 4 A and B. The black dots represent individual readings of root length. It will be seen that they both exhibit the typical S-shaped curve and that the period of maximum growth rate (grand period of growth) is attained very soon after the emergence of the radicle and is maintained for many days before the growth rate declines at the top of the curve. From this point onwards, the growth in length of the main root continues at a rate of the order of a tenth that in the grand period of growth. The duration of this grand period of growth varies considerably from individual to individual (see Fig. 4 A, and also later Fig. 8).

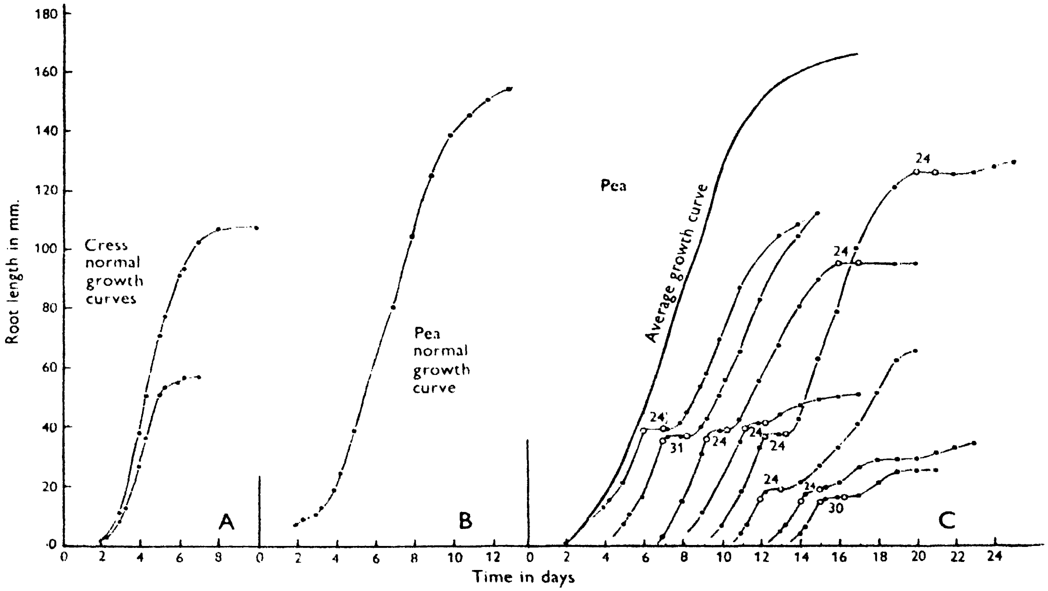


Fig. 4. Growth curves of cress and pea. A. Normal growth curves of cress. B. Normal growth curve of pea. C. Growth curves of pea with interpolated inhibition periods of approximately 24 hr. induced by 1 p.p.m. 2:4-D. The actual times of exposure in hours are marked in the relevant curves.

In cress, lateral roots do not make their appearance until this final low level of growth rate of the main root is reached, and experiments on this species have not been extended for periods longer than this. In the pea, on the other hand, lateral roots appear usually at a time when the grand period of growth is only two-thirds completed. The termination of the grand period of growth is scarcely due to such causes as the exhaustion of essential nutrients since, under the given experimental conditions, such material should be present in excess. Continued growth of lateral roots emphasizes this conclusion. It might be suggested that, just as terminal buds inhibit the growth of laterals, so the development of lateral roots might bring about an inhibition of the growth of main roots, either by diversion of essential raw materials for growth or by some type of hormonic control. A discussion of these possibilities will not be undertaken here but, for the time being, termination of the grand period of growth will be regarded as an attribute of the age of the apical meristem and due, it will be presumed, to maturation processes taking place in that tissue.

RESULTS WITH 2:4-DICHLOROPHENOXYACETIC ACID

(*a*) *The general nature of* 2:4-D. *action*

In Fig. 4C has been collected a series of growth curves for pea roots, treated during the grand period of growth with 1 p.p.m. 2:4-D. for periods of about 24 hr. The origins of these curves, i.e. the times of soaking of the seeds, are spread out along the time axis to facilitate drawing and to avoid undue confusion of points. The smooth curve to the left of the group is a mean curve of normal growth constructed from a number of curves from untreated pea seedlings. In these curves the first small circle represents the time of application of the solution of growth substance and the second small circle the time of its replacement with fresh culture solution. The time interval between these two points is recorded by each graph.

It will be seen that immediately after the application of this concentration of growth substance the growth rate of the root decreases rapidly and, after a period of a few hours, reaches a new rate scarcely distinguishable from zero in these progress curves. On removal of the external supply of growth substance and replacement with fresh medium this low rate of growth persists for a time, but, after a lag phase of varying duration, growth is again resumed and may, in many cases, attain a rate as great as the normal in the grand period of growth. The growth rate eventually declines to a final steady low value as in normal growth. The striking feature of those curves showing a complete recovery of the normal growth rate is that the sole effect of the growth substance seems to be merely an interpolation in the normal growth progress curve of a quiescent period, limited in its duration only by the continued presence of the growth substance in the surrounding medium, no other deleterious effect on the subsequent root growth being detectable. In other cases, however, the recovery is not so complete, and an inhibition persists long after the removal of the external source of inhibitor. During this recovery period, however, lateral roots generally appear from the treated portion of the radicle in numbers much greater than normal, due to the stimulation of meristematic activity of the pericycle cells.

This dual effect of growth substances has long been known from the researches of many workers on the subject. The first, an inhibition of longitudinal growth, acts on the apical meristem or on the elongating region immediately behind. The second, a remarkable stimulation of meristematic activity of the cells of the pericycle, acts in all those parts of the radicle receiving growth substance. (For a general review see Thompson, 1945; also Delarge, 1941.) Innumerable lateral root meristems are initiated but do not grow out, since longitudinal growth is inhibited in them also. On removal of the inhibitor longitudinal growth is resumed both in the main root and in these lateral roots after a varying time lag.

Pl. 4 shows a series of photographs taken at 24 hr. intervals of a seedling of ***Pisum sativum***, illustrating this phenomenon (A, 1–8). No. 1 is of the normal seedling 3 days after soaking and at the time of addition of a solution of 10 p.p.m. 2:4-D. No. 2 shows the seedling after 24 hr. exposure to this solution and at the time of washing and replacement of the medium by fresh culture fluid. The complete inhibition of longitudinal growth is clear (normal growth would have doubled the length of this root in this time) together with the typical swelling due to lateral root meristem activity.

The subsequent photographs illustrate the recovery phenomena. No. 3 shows clearly the renewal of growth activity of the main apical meristem after 24 hr. and also the continued lateral swelling. In No. 5 (3 days from washing) the whole cortex of the treated portion has split off as a result of the excessive production of lateral meristems and can be seen on the left, with the central stele, and its rows of tooth-like lateral meristems, bulging out to the right. Subsequently, these lateral meristems grow out as solid vertical sheets of tissue above the protoxylems of the main stele. The cortices of these laterals are fused but the central steles remain separate. This results in a most remarkable root fasciation effect. In the last two photographs the sloughed cortex, seen on the left in Nos. 5 and 6, has slipped round the back between two such sheets of lateral roots. This is essentially a recovery phenomenon since, as would be expected, there is no growth outwards of lateral roots if the inhibiting concentration of growth substance is maintained in the surrounding solution. This is well illustrated in Pl. 4, B, which is a photograph of a pea root removed from the seedling after growth for 8 days in a solution containing 1 p.p.m. of 2:4-D. Here the whole cortex has sloughed off.

These morphological effects will not be considered in any detail and the results which follow deal only with the inhibition of the longitudinal growth of main radicles, i.e. an action on the main apical meristem and adjacent elongating zones.

(*b*) *Root growth in* 2:4-D.

It has been shown in numerous researches that, if roots are grown continuously in various concentrations of growth substances, a definite relationship exists between the longitudinal growth rate and the concentration. Thus in very low concentrations of the order of 1 part in 10^{10} it has been claimed that the growth rate is stimulated (see p. 196). In higher concentrations, however, there is a definite inhibition and it is found that the degree of inhibition is roughly proportional to the logarithm of the concentration of the growth substance in the medium surrounding the root. This is illustrated in Fig. 5, where results of a large number of growth experiments with cress seedlings are collected together (for description of methods see Audus & Quastel, 1947*a*). The lengths of the vertical black lines in this graph represent twice the standard error of the mean of a number of measurements of root-growth inhibition and are plotted on the mean itself as a mid-point. The relevant numbers of observations are marked below each line. These measurements represent the average inhibited growth rate (rate of increase of fresh weight) over a period of 7–10 days, from germination onwards. It would seem that the relationship between percentage inhibition and log concentration is not strictly linear but the data do not warrant any attempts to evaluate a more precise empirical relationship. A free-hand curve has been drawn through the points and extrapolation gives approximate upper and lower concentration limits for 2:4-D. inhibition of 3·7 and 0·035 p.p.m. respectively. (Experiments showed no inhibition in a concentration of 0·01 p.p.m. and 100% inhibition in 10 p.p.m. (see circles on graph of Fig. 5).)

These results are for average growth rates over considerable periods. It would be interesting, however, to follow the rates of change of growth rate of roots on subjection to the action of the inhibitor, as, for example, during the inhibition periods already described. It has been seen from Fig. 4 C that the growth rate falls away rapidly in the presence of the growth substance and finally reaches a low, although usually measurable, value. The technique previously described was not sufficiently accurate to study these slow growth rates over short time intervals, especially with cress, where the datum point at the junction of root and hypocotyl might be gradually descending in the apparatus. Using pea, therefore, a new technique was adopted in which the growth was followed by means of a graduated scale in the eyepiece of the travelling microscope and changes in root length could then be measured with an accuracy of 0·01 mm. In pea, root growth could be accurately determined from the rate of descent of the tip and was not complicated by any appreciable shift of the root base over the duration of the experimental period.

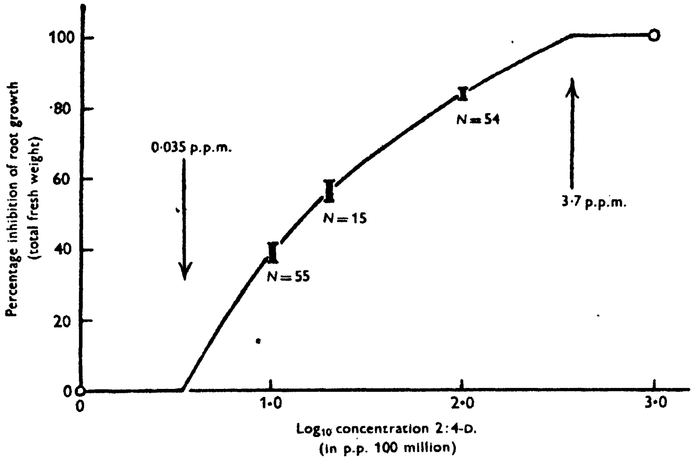


Fig. 5. Graph showing the relationship between inhibition of root growth (average over a 7–10-day period) and concentration of 2:4-D. in the water-culture medium. (For further explanation see text.)

By this method hourly observations were made on root growth rate during the first few hours in the growth substance and subsequently over longer periods as the rate became very low. A wide range of concentrations of 2:4-D. was studied and a number of curves obtained for each concentration. The normal growth rate was followed for 2 or 3 hr. previous to the addition of the growth substance and these values, together with the value obtained from the slope of the main progress curve (see Fig. 4), were combined to give a mean estimate of the normal growth rate. It might be argued that inhibitions should be estimated on a basis of the growth rate immediately prior to the application of the inhibitor. In view of the fact, however, that the rate of inhibition is slow in low concentrations and may take many hours to reach a value of 50%, it is impossible to say how far small diurnal rhythms may affect the apparent rate of inhibition. In this case the most accurate basis on which to estimate the inhibition is the average normal growth rate. A typical inhibition progress curve is shown in Fig. 6A, for a concentration of 10 p.p.m.

2:4-D. It will be seen that the inhibition attains a value of the order of 50% within the first hour, but that the final very low rate is not reached until after a very much longer period. Smoothed curves from the average of a number of such stepped curves have been

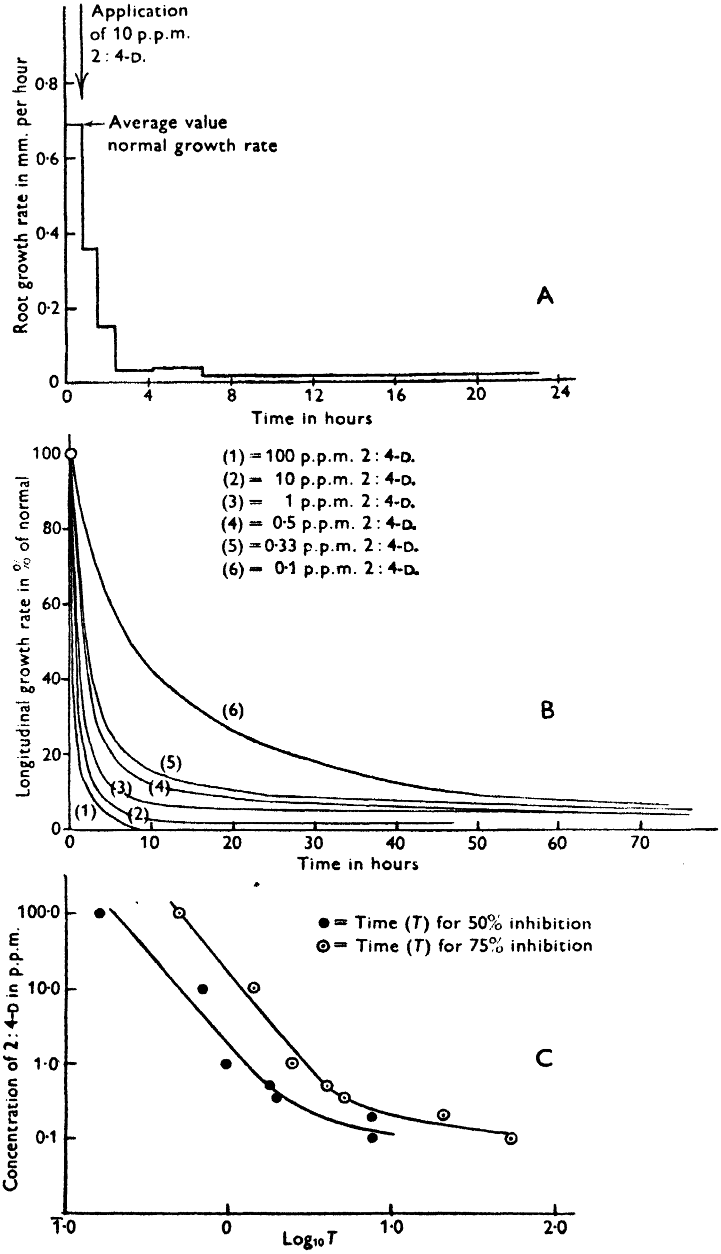


Fig. 6. A. Typical curve of growth deceleration on submitting a pea root to an inhibiting concentration of 2:4-D. B. Smoothed average curves of growth deceleration of pea roots on exposure to various concentrations of 2:4-D. C. Analysis of the curves in B. The relationship between growth deceleration and external concentration of inhibitor.

drawn for each concentration of growth substance. These curves appear in Fig. 6B. It will be seen that the rate of inhibition is directly correlated with the growth substance concentration. In addition, it would seem that a final steady level of inhibited growth rate is not attained, but that the growth rate continues to fall slowly even after 3 days' inhibition. All these experiments were carried out early on in the period of constant growth rate, when maturation phenomena (see p. 202) would be expected to be at a minimum. In the highest concentration (100 p.p.m.) growth ceases completely after about 9 hr.

The question now arises as to whether or not this progress curve of inhibition is a direct reflexion of the rise of internal concentration of growth substance, i.e. is conditioned by the permeability of the root cells to the growth substance. Albaum, Kaiser & Nestler (1937) have found that, in the intake of β-indoleacetic acid by *Nitella* cells, entry follows the laws of simple diffusion, i.e. the rate of entry is proportional to the concentration gradient. They also find that the final external and internal concentrations are equal. That being the case here, we should expect the rate of inhibition of root growth to be proportional to the external concentration of growth substance. To elucidate this point the whole series of curves for various concentrations of growth substance have been subjected to analysis. From the smoothed curves the times (T) for the inhibition to reach 50% and 75% have been calculated and taken as a measure of the rate of inhibition. These values show a definite relationship with the concentration and they range from 10 min. (50% inhibition in 100 p.p.m.) to 21 hr. (75% inhibition in 1 part in 10 millions). In order to compress the time and concentration scales to allow of easy graphic representation the values of the logarithm of these two times, viz. for 50% and 75% inhibition ($=\log_{10}(T)$), have been calculated and plotted against a logarithmic scale of concentration. These graphs appear in Fig. 6C.

One striking characteristic of these two graphs is that for concentrations from 100 to 0·33 p.p.m. the relationship between $\log_{10}(T)$ and $\log_{10}$ (concentration) is, within the limits of experimental error, linear. In addition, the two curves for 50% and 75% inhibition run almost parallel. From the slope of these two curves it would appear that T is inversely proportional to (concentration)$^{0\cdot4}$, i.e. the rate of inhibition ($\propto 1/T$) is directly proportional to this power of the concentration. As the concentration falls, however, the rate of inhibition tails away and, over the concentration range 0·1–0·33 p.p.m., becomes more nearly proportional to (concentration)$^{1\cdot0}$. It is obvious that in much lower concentrations, where there are very small or no inhibitions, the value of T tends to infinity and no longer is a measure of the rate of inhibition.

Thus it seems reasonable to suppose that in the lowest concentration range (0·1–0·33 p.p.m.) the rate of entry of growth substance may be controlling the rate of inhibition, since both are proportional to the external concentration, i.e. the concentration gradient (cf. Albaum *et al.* 1937). In the higher concentrations, on the other hand, the rate of inhibition is controlled by some other factor. In this respect it is interesting to compare the relationship

$$\text{(Rate of inhibition)} \propto \text{(concentration of growth substance)}^{0\cdot4}$$

with the Freundlich adsorption isotherm

$$\text{(Solute adsorbed per unit of adsorbent)} \propto \text{(concentration of solute in solution)}^{K/n}$$

which has the same mathematical form. It is tempting to suggest that at these concentrations, therefore, the rate of inhibition of growth is proportional to the amount of growth substance adsorbed on some colloidal interface within the cell.

The percentage inhibition of root growth, as it appears in Fig. 5, presumably gives a measure of the final equilibrium value to which such growth deceleration curves as those of Fig. 6B approximate. The two sets of results are not strictly comparable. Those of Fig. 5 are for growth in fresh weight, whereas those of Fig. 6 are for growth in length. The former are therefore complicated by other growth-substance effects, i.e. stimulation of lateral root meristems, swelling of cortical cells, etc. However, experiment has shown that the general form of the inhibition-growth-substance-concentration relationship differs little with the method of growth measurement, and it seems therefore that the average degree of inhibition measured over a long period of time bears a relationship to inhibitor concentration that is different from that of the rate of inhibition immediately following application. This must be partly due to the fact that the former value is an integration, over a period of time, of a number of growth-substance effects, direct and indirect, which may show marked drifts with duration of exposure to the inhibitor. These time drifts will be considered in the following sections.

(*c*) *Recovery from inhibition*

Reference to the curves of Fig. 4C shows that there are at least three phases in the growth curves after removal of the inhibitor (2:4-D.). In order of appearance they are:

(1) A lag phase, of variable length, during which the rate of growth gradually increases to a maximum in

(2) a period of constant growth rate;

(3) a final tailing off of the growth rate corresponding with that found at the top of the normal S-shaped curve.

The character of this first phase may serve as an indication of the rapidity with which the inhibitor is removed from the site of its action in the root. It may, on the other hand, be a result solely of the gradual restoration of the normal metabolic equilibrium so grossly disturbed over the period of the inhibition, the inhibitor having been removed within the first few hours of washing. In the second phase the maximum slope of the growth curve indicates the degree to which the root has recovered its normal growth activity. The onset of phase three is presumably an indication of the degree of maturation of the root, as in the case of the normal growth curves.

In an attempt to explore the main controlling factors of these three phases and to elucidate, as far as possible, the nature of this reversible inhibition, a large number of observations were made using a wide range of concentrations of 2:4-D. for varying periods of time both with garden pea (*Pisum sativum*) and with cress (*Lepidium sativum*). As in the case of experiments illustrated in Fig. 4C, exposure of the roots to 2:4-D. was usually started early in the grand period of growth, i.e. about 5 days after soaking the seed, when the maximum growth rate had usually been attained. The actual time varied somewhat, owing to the varying rapidity with which the seeds germinated but, as far as possible, inhibition periods were commenced when roots had reached roughly the same length (i.e. 30–40 mm.).

The results of all experiments on pea are collected in Fig. 7. In order to simplify presentation and to facilitate comparison of curves for different treatments, only those portions of the growth curves subsequent to the commencement of inhibition have been

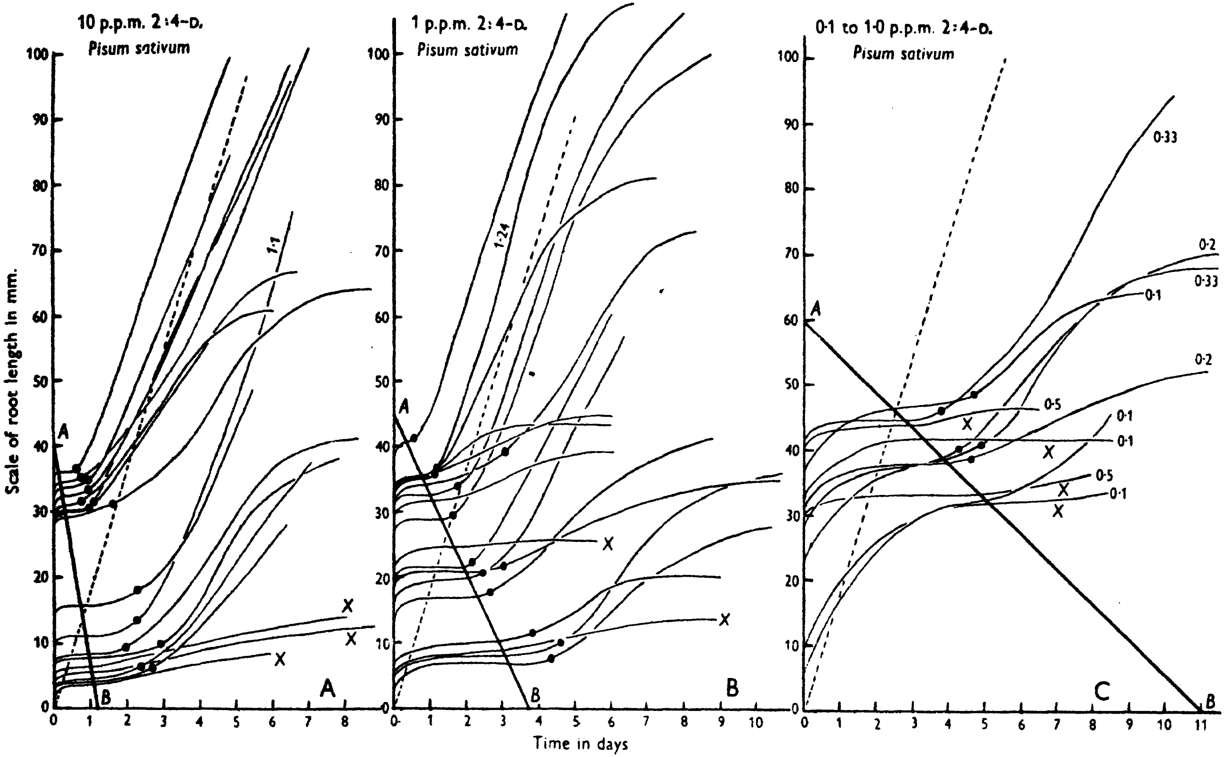


Fig. 7. Collected growth curves for pea roots during and subsequent to varying periods of exposure to a range of concentrations of 2:4-D. The figures against the curves in the right-hand section give the relevant inhibitor concentrations in p.p.m. (For further explanation see text.)

drawn. All curves have been drawn so that the time of commencement of the inhibition is at zero. The curves have now been ranged vertically so that the time of removal of the inhibitor falls on the thick line *AB*, drawn obliquely across the corner of the graphs. The distance from the zero time axis to the point of intersection of any curve with this line *AB* is equal to the time of exposure to the inhibitor. This time increases uniformly as we descend from top to bottom of any one series. The dotted lines in the graphs give the maximum slope of the average normal growth curve under these experimental conditions.

Let us first consider from these curves the effect of concentration and duration of exposure on the final degree of recovery of growth, as indicated by the maximum growth rate attained after washing. In a few cases recovery is very slight or nil (graphs marked *X*) and it would seem that in these cases some irreversible autolytic process must have set in during inhibition. The occurrence of these latter inhibitions seems to bear no obvious relation to concentration. They do not occur, however, for inhibition periods of less than 24 hr. This would accord with the view that after 24 hr. a toxic metabolic product may have accumulated to such an extent that a persistent growth inhibition is the result. Where inhibition is reversible, either this toxic product accumulates at a much slower rate, or the critical concentration for persistent inhibition is not reached.

In the majority of cases, however, when recovery has taken place, a maximum growth rate approaching that of the normal root is attained. There is some indication from the graphs that long inhibition periods are correlated with lower maximum growth rates after recovery, but this may be due to an early tailing-off of the growth curve after the lag phase. This is particularly obvious in the third set of curves for the lowest concentrations (1–0·1 p.p.m.). The most important feature of the curves, as shown by the similarity of the two sets for 10 and 1 p.p.m., is the lack of any obvious relation of the recovery characteristics to the concentration of inhibitor. These curves also show that there is no evidence of a stimulation of growth rate after reversal, as claimed by Thimann & Lane (1938). Only in two cases were growth rates observed having values above the average normal growth rate, giving ratios to the normal of 1·10 and 1·24. These ratios are marked against the respective graphs in the figures and are well within the limits of sample variation.

In order to obtain a measure of the lag in these curves, a position has been determined at which the recovery curve attains a slope equal to half the maximum slope. This was not possible in all cases, due either to premature discontinuance of observations or to a small degree of total recovery. Wherever possible this point has been marked by a black dot on the curves. The distance of this dot from the oblique datum-line of washing times gives a measure of the lag.

Comparisons of the three sets of graphs show that both concentration of inhibitor and duration of the exposure period affect the length of the lag period. Thus results for both 10 and 1 p.p.m. show an increase in the lag as the length of exposure to inhibitor is increased. In addition, the lags for comparable exposure times in 10 p.p.m. are considerably longer than in 1 p.p.m. and less. In spite of the large scatter it would seem that there is little effect of concentration over the range 1–0·1 p.p.m. In all concentrations, the longer the exposure time the longer is the lag. This would support the view that the rapidity of recovery is inversely correlated with the degree of dislocation of the normal dynamic growth equilibrium. It seems feasible to suppose that once a critical degree of derangement or a critical concentration of toxic metabolic products had been reached then the whole process would become irreversible. Up to this critical point growth can be recovered completely, but the time taken for this recovery is directly related to the extent to which this critical point has been approached.

It is relevant here to mention recent observations on the inhibiting effects of small concentrations of naturally occurring amino-compounds on longitudinal root growth. Such compounds as glycine, for example, have been shown to bring about inhibitions at concentrations as low as 100 p.p.m. (Audus & Quastel, 1947*b*). It is not inconceivable that such compounds might accumulate in the root cell during the inhibition period, and it is obvious that direct observations on the fluxes of normal metabolites during this inhibition is the next line of approach to this problem.

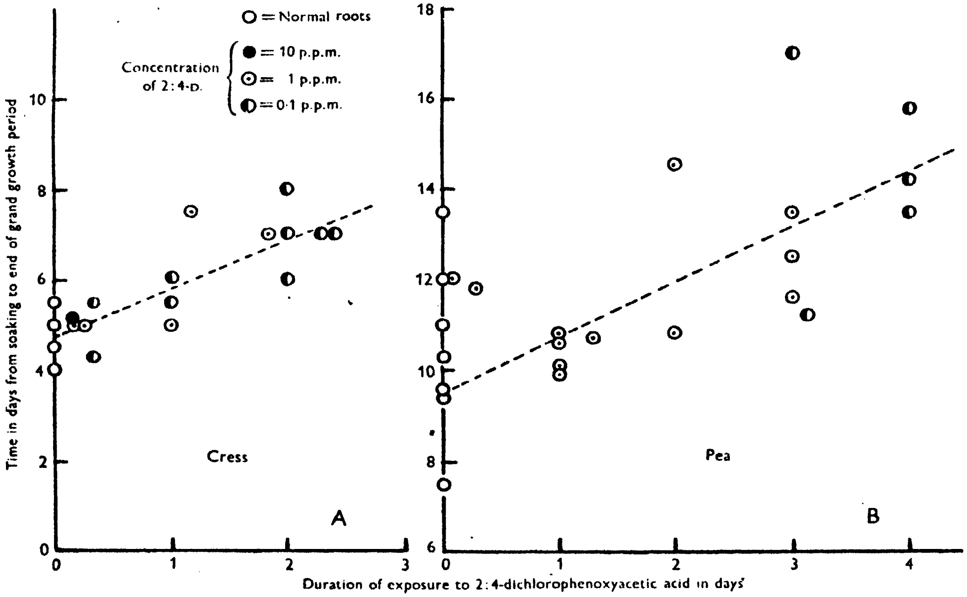


Fig. 8. Graphs showing the effects of varying periods of exposure to 2:4-D. on the duration of the grand period of growth in pea and cress roots.

The difference in the observed relationships of growth-substance concentration to initial inhibition rate and to average inhibition over a 7–10-day period (see p. 207) is probably largely attributable to time effects such as these.

The effect of high growth-substance concentration (10 p.p.m.) in increasing the lag might be due to an accelerating effect of the high inhibitor concentration on the rate at which the cell equilibrium is disturbed, or it might be due to the longer initial time necessary for the complete removal of the inhibitor by washing. This can be decided only by more direct experimentation.

As was noted on p. 209 there is an indication that long exposures to the inhibitor seem to give rise to smaller degrees of recovery, as indicated by the maximum growth rate attained after reversal. It was suggested, however, that this might be due to the onset of maturation causing a tailing-off of growth, as occurs under normal conditions in untreated seedlings. To investigate this point, the time of onset of this tailing-off period has been determined in all curves where possible. This has been taken as the time from soaking of the seeds to that point on the growth curve where the final falling-away from the maximum is just detectable. Results for pea are seen in Fig. 8B, where this time is plotted against the duration of exposure to 2:4-D. It will be seen that, in spite of the high degree of scatter, there is a direct and significant correlation between time of exposure and time of onset of this decrease in growth rate. What is more striking, however, is that the slope of the straight line of nearest fit is very nearly unity (actually 1·22 in the case of pea), which means that the delay in the onset of this phase is, to all intents and purposes, equal to the time of exposure to the inhibitor. From this it is logical to deduce that, if we attribute the final fall in growth rate at the end of the grand period of growth to maturation processes taking place in the root meristem, then these maturation processes are also inhibited by the action of the growth substance and are resumed again when the inhibitor is removed.

It appears, therefore, that the full recovery of growth rate after these long inhibition periods is not limited by the onset of the maturation phenomenon. Such a limitation can, to a certain extent, be accounted for by an excessively long lag phase after long exposures. It is much more feasible, however, that these smaller growth rates are due to persistent inhibitions remaining after removal of the growth substance, and due to a permanent partial dislocation of the cell growth mechanism brought about by an accumulation of toxic metabolites above the critical concentration for complete reversal. Much more work is needed to bring about full elucidation of these points.

(*d*) *Results with cress*

Fewer experiments have been carried out with cress, owing to the difficulties mentioned in the descriptions of technique. The results of all experiments to date are collected in Fig. 9.

No accurate observations were made on the rate of inhibition of growth, but a glance at the two sets of curves will show that they resemble, in the main, those obtained with pea. Here, too, the rate of inhibition in the higher concentration (1 p.p.m.) is more rapid than in the lower (0·1 p.p.m.). Comparisons with Fig. 7 show that in this respect cress is much more sensitive to 2:4-D. than pea. Rough estimations from these curves of Fig. 9 give the average time for the attainment of 75% inhibition of growth as 1·7 hr. in 1 p.p.m. and 4·2 hr. in 0·1 p.p.m. For pea the corresponding values are 2·4 and 53 hr. It is interesting also to compare the ratio of these two times for cress (i.e. $4·2/1·7 = 2·47$) with that expected from the relationship deduced for pea on p. 206. The expected ratio from the expression is equal to (ratio of concentrations)$^{0·4} = 10^{0·4} = 2·51$, a remarkably close agreement considering the relatively large errors involved in the estimations. Thus it would seem that over this concentration range (i.e. 1–0·1 p.p.m.) the same relationship between inhibition rate and concentration of inhibitor exists as in the concentration range 10–0·33 p.p.m. for pea.

When we turn to the recovery of growth after inhibition we see similar features in the results of cress and pea. With cress we find a rather large number of individuals that show little or no recovery and these again occur only with inhibition periods of over 24 hr. The degree of recovery (maximum slope of growth curves after removal of inhibitor) tends to be complete for short periods of inhibition, but becomes rapidly smaller as the exposure time is increased. The maturation effect was again considered here and results are shown in Fig. 8A. Here again the same correlation is observed between extension of the grand period of growth and the duration of exposure to the growth substance, and the slope of the line of nearest fit approximates even more closely to unity (actual value 1·04). Thus here also in cress the growth substance would appear to be inhibiting maturation processes as well as growth in length of the root. Again maturation processes would seem to be playing little or no part in causing the apparent rapid deterioration, with increasing exposure, of the capacity of the cress root to recover from inhibition. Comparison of Fig. 9 with Fig. 7 stresses the greater sensitivity of the root-growth mechanism in cress to long-continued dislocation of the normal metabolic equilibrium. In addition there is again no evidence of a stimulation of root growth after reversal of inhibition.

One striking difference between the results from cress and those from pea is the relative absence of the lag in the recovery curves of cress. This may be due to the relatively greater ease with which the inhibitor is removed from the more slender roots of the cress but, in the light of our conclusions drawn from the pea experiments, it is unlikely that this is the whole story.

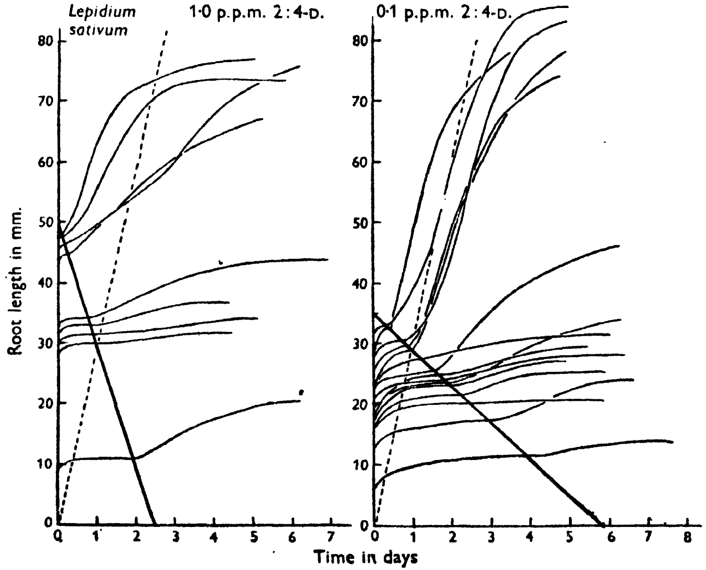


Fig. 9. Collected curves of growth of cress roots during and subsequent to varying periods of exposure to two different concentrations of 2:4-D. (For further explanation see text.)

(e) *Discussion of results with 2:4-dichlorophenoxyacetic acid*

The general picture which can be formed from the results presented in the foregoing sections can be briefly summarized as follows. The growth substance enters the cells of the growing region of the root by a simple process of diffusion. Once in the cell, molecules are adsorbed on to some colloidal surface upon or from which their inhibitory action is exerted. In low concentrations the rate of onset of inhibition would seem to be limited by the rate of entry of the growth substance into the root cells, but at higher concentrations it becomes proportional to the number of adsorbed molecules. Over the period of growth inhibition it seems that physiological ageing of the meristem cell is also to a large extent retarded. The full extent to which the total metabolic complex is affected by this inhibition can be but vaguely imagined, but it is highly probable that many processes do continue at relatively unimpaired rates. The resulting steady dislocation of the normal dynamic growth equilibrium is completely reversible if the inhibitor is removed within certain time limits. Beyond this limit the accumulation of certain metabolic products (e.g. amino-acids, see Audus & Quastel, 1947*b*) presumably bring about a persistent partial inhibition of growth, which increases in degree as the concentration of these products increases, i.e. as the inhibition period is extended. Finally, a critical concentration is reached beyond which all growth potentiality is lost.

In view of these facts it would seem that the term 'phytocidal', which has of recent years been applied to this growth-inhibiting action of growth substances, is a misnomer. It is suggested that instead the term 'phytostatic' should be adopted as a more precise description of these reversible inhibitions of root growth.

RESULTS WITH COUMARIN

It was demonstrated in a previous paper (Audus & Quastel, 1947*a*) that coumarin, a naturally occurring metabolite in many species of plant, exhibits physiological properties that are similar to those of the growth substances. The most important of these properties is the inhibition of longitudinal root growth, a phenomenon which, like that of auxin action, is reversible. In addition, certain formative effects produced by coumarin on seedlings are very similar if not identical with those of the auxins, e.g. hypocotyledonary swellings, rooting of cuttings and lateral root stimulation in seedlings (Audus, unpublished work). It has also been shown that, running parallel with these morphological effects, coumarin has marked effects on mitosis (Cornman, 1946, 1947). Heteroauxin is also known to have marked effects on chromosome morphology (Levan, 1939). It differs from the growth substances, however, in showing marked powers of inhibition of seed germination in relatively low concentrations, a phenomenon seen in the growth substances only in relatively high concentrations (Audus & Quastel, 1947*a*).

Preliminary experiments have already shown that both the inhibition of the longitudinal root growth and also of germination are reversible, provided the exposure to the coumarin is not of too long a duration. Recently further experiments have been carried out along the same lines as those already described for 2:4-D., in order to see how close this parallelism of root-growth inhibition might be. A range of concentrations as wide as those used for 2:4-D. could not be employed here, since limits are set by the smaller effectiveness of coumarin on the one hand (no significant inhibition of root growth with 1 p.p.m.) and its solubility on the other (saturated solution approximately 100 p.p.m.).

Two sets of results for pea are collected together in Fig. 10A and B, where progress curves of root length are drawn during and after exposure to two concentrations of coumarin (i.e. 100 and 30 p.p.m.) as in Figs. 7 and 9. No observations have been made on the rate of onset of the inhibition by the accurate technique previously described, but it can be seen from these two sets of curves that growth deceleration on the application of coumarin is correlated with the concentration of inhibitor. Determinations of the time taken for growth to reach 25% of normal have been made from the slopes of these graphs. The values obtained were 7·93 ± 0·99 hr. for 100 p.p.m. and 17·25 ± 2·68 hr. for 30 p.p.m. These figures show that the deceleration of growth rate in coumarin is much slower than that in 2:4-D. with solutions of comparable concentrations, 100 p.p.m. 2:4-D. bringing

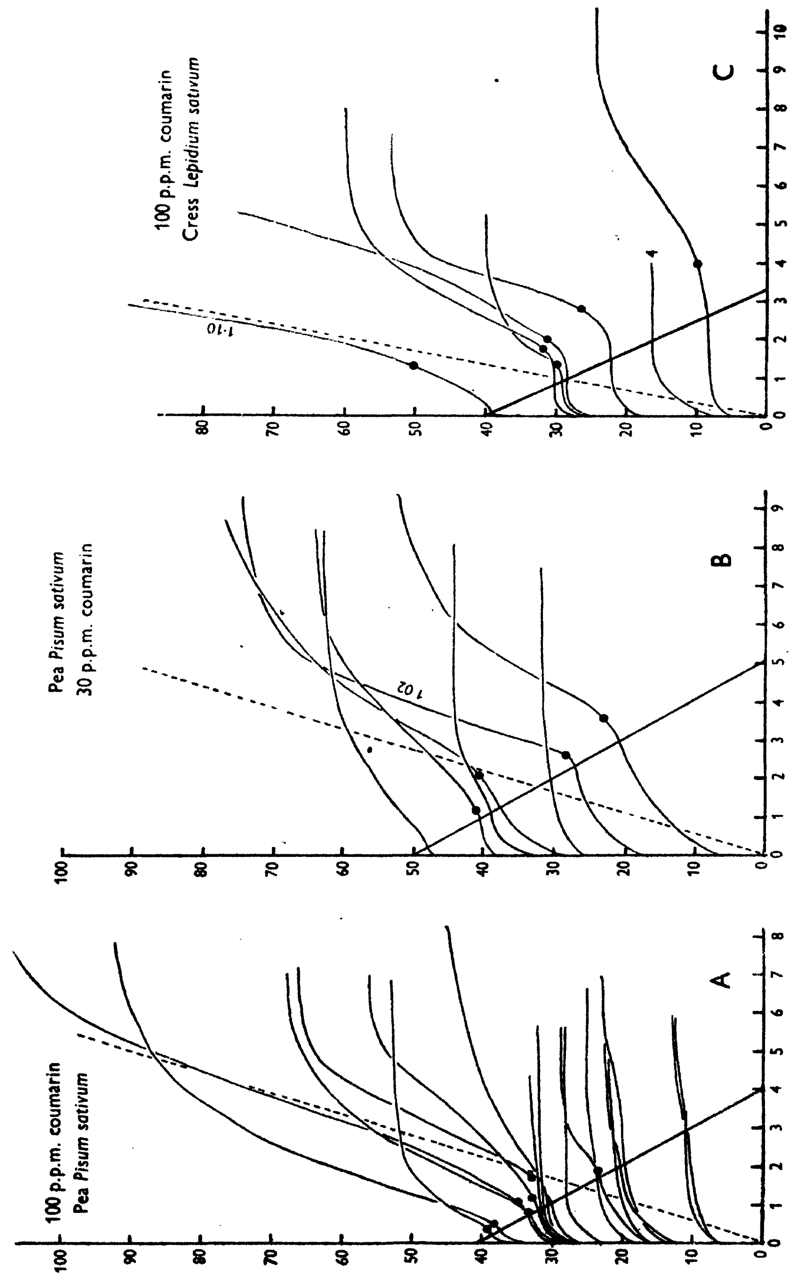


Fig. 10. Collected curves of growth of pea and cress roots during and subsequent to varying periods of exposure to coumarin. (For further explanation see text.)

about 75 % inhibition of root growth in half an hour as compared with the above value of nearly 8 hr. in the case of coumarin. In addition, the figures suggest that the rate of deceleration is proportional to the external concentration as in low concentrations of 2·4-D., a reduction of the concentration to 30 % reducing the growth deceleration to just under a half (0·46). It, therefore, seems likely that over this concentration range the rate of entry of coumarin into the cell (presumed proportional to the external concentration of coumarin, i.e the concentration gradient) is very largely limiting the growth deceleration.

A few observations have been made on cress (see Fig. 10C), where a value of 5·0 ± 1·3 hr. for 75 % inhibition in 100 p.p.m. was found, thus showing a slightly greater rate of inhibition than in pea. This is correlated with a much more slender root and may be due to shorter time necessary for coumarin to spread over the whole cross-section of the root meristem.

Turning to the curves of growth after removal of the external source of coumarin, the following points of comparison with the 2:4-D. curves can be made.

(1) A far greater number of roots show no growth subsequent to treatment with coumarin, although here again no such irreversible action occurs for periods under 24 hr. This occurs most frequently at the higher concentration of 100 p.p.m. It seems therefore that coumarin itself, in addition to its phytostatic action, may have a cumulative irreversible action on the root cells, which may be direct (i.e. a toxic action of the coumarin molecule or products of its decomposition in the root) or indirect (accelerated accumulation of toxic metabolic products). In the case of cress this toxic action is not so apparent, the recovery curves comparing favourably with those of 2·4-D. in Fig. 9.

(2) Where possible the same measure of the lag has been determined and marked as black points on the curves. These few points show that on the whole this lag is much shorter for coumarin inhibition in pea than for 2:4-D. inhibitions. On the other hand, in cress the reverse is the case and the significance of these differences is very obscure. However, although the concentration range is much smaller it would seem that for the few points available from the results on pea, there is little effect of concentration of inhibitor on this lag.

On the whole, therefore, these results are capable of explanation along the same lines as those for 2:4-D., namely, that the main factor controlling the rate of recovery from inhibition is the extent of the dislocation of the normal growth equilibrium. Concentration plays only an indirect part in setting the level of this dislocation.

(3) The full extent of the growth recovery, as indicated by the maximum slope of the growth curve after washing, would seem on the whole to be as complete as that for 2:4-D. inhibition. As in the case of 2:4-D. there is no evidence here of a stimulation of root growth above normal after recovery. In the one case where a greater growth rate was observed (see Fig. 10C) the ratio (1·10) was within the limits of normal sample variation.

(4) There is, however, a greater proportion of curves which show a small total growth after washing. To investigate the possible part played by maturation phenomena in these recoveries, the times of onset of this third phase in the growth curve have been determined and plotted against duration of exposure to inhibitor as in Fig. 8. These results for coumarin and pea appear in Fig. 11. In contrast to the results for 2:4-D. there is no increase in the length of the grand period of growth correlated with the duration of the period of inhibition. Instead, points for 100 p.p.m. coumarin suggest that, if anything,

exposure to this concentration results in a decrease in this period of maximum growth rate, i.e. an acceleration of the so-called maturation processes in the root. Low concentrations (30 p.p.m.) show no significant effect on this grand period of growth. These facts make it quite probable that in the case of pea the smaller total growth recovery after coumarin inhibition as compared with results for 2:4-D. is due very largely to the onset of normal maturation and its correlated fall in the growth rate. The case of cress will not be considered here as too few values have so far been obtained.

In the course of these experiments it was demonstrated that lateral root production in pea is also stimulated by coumarin as it is by 2:4-D. (see p. 202). Longitudinal growth inhibitions not being so complete in this case, some lateral roots continue to grow out, although at a slow rate, and present the appearance shown in Pl. 4. Here a series of photographs of a pea seedling have been taken at intervals of 48 hr. during exposure to a nutrient solution containing 50 p.p.m. of coumarin (C 1–7). They show the inhibition of longitudinal growth on the main root and the progressive lateral swelling associated with enlargement of cortical cells and the activity of lateral root meristems in the pericycle. This same swelling phenomenon is repeated in the laterals that grow out. These morphological effects are at present being investigated in more detail and it is hoped will form the subject of a future communication.

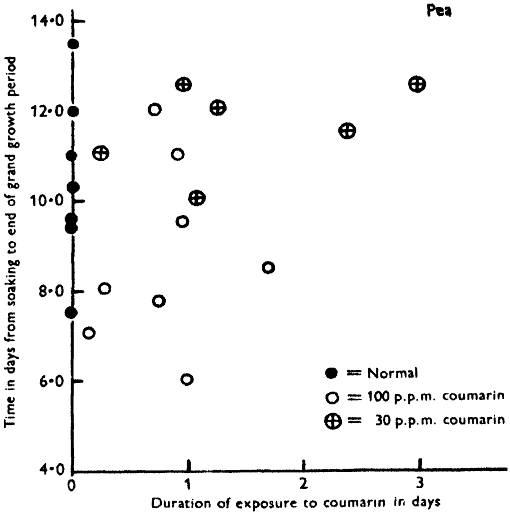


Fig. 11. Graphs showing the effect of varying periods of exposure to coumarin on the duration of the grand period of growth in pea roots.

GENERAL DISCUSSION

A general comparison of the foregoing results indicates that longitudinal root-growth inhibitions brought about by low concentrations of 2:4-D. and coumarin are very similar. In both cases, from studies on the kinetics of the inhibition, indirect evidence indicates that at low physiological concentrations the rate at which growth is retarded may be limited by the rate of entry of the inhibitor into the cell. At higher concentrations of 2:4-D. indirect evidence again suggests that the inhibitor is adsorbed at some colloidal cell interface, upon or from which its action is effected. This is in accord with recent work by Veldstra (1944) on the surface activity of plant-growth substances. With coumarin, relatively low effectiveness of the molecule and low solubilities precluded similar kinetical studies in high concentrations and thus prevented further comparisons with 2:4-D. action.

In both cases the degree of recovery from inhibition seems determined almost entirely by the length of exposure to the inhibitor and is relatively independent of its concentration. This can be explained by the assumption that the direct growth inhibiting action of these two molecules is completely reversible. This is made evident by the complete recovery of normal growth rates after inhibitory periods up to 24 hr., and supports the previous suggestion of a simple easily reversed adsorption of the growth substance on to some colloidal interface.

As previously outlined however, this inhibition, which will not extend to all metabolic processes in the cell, will result in a progressive displacement of normal dynamic cell equilibrium, which itself becomes less susceptible of reversal as the inhibition period is extended. This encroachment of irreversible autolytic components into the total complex of cell metabolic processes is secondary to the main action of the inhibitors and therefore (theoretically) independent of their concentration. This seems to be rigidly true in the case of 2:4-D., but with coumarin there is an indication that high concentrations (100 p.p.m.) tend to accelerate these secondary irreversible inhibitions. No doubt this is correlated with the much higher concentrations of coumarin necessary to produce the same degree of primary growth inhibition.

A normal meristem, growing under the conditions described, shows a definite ontogenetic drift in its activity as illustrated by the longitudinal growth rate of the main root. This drift may be intrinsic (i.e. an ageing or maturation phenomenon) or directly conditioned by the availability of growth factors (e.g. raw materials for synthesis, vitamins, hormones, etc.). There is strong evidence that this drift is also affected by 2:4-D., the termination of the period of maximum growth rate (commencement of the upper hook of the S-shaped growth curve) being delayed to an extent commensurate with the duration of the inhibition period. Whatever their exact nature, however, it would seem that these 'maturation' processes in the root meristem are also inhibited by 2:4-D. action. Coumarin, on the other hand, seems to accelerate them in high concentrations; a result which is in general agreement with its suggested augmentation of the irreversible secondary growth inhibitions outlined above. These are probably both expressions of a secondary action of the coumarin molecule acting in high concentrations (cf. the germination-inhibiting properties of coumarin which are not shared by growth substances except at very high concentrations).

It is becoming more and more obvious, as new facts concerning the responses of plants to growth substances are brought to light, that their action in the complex phenomena of growth is a multiple one. It has been thought that all such phenomena might be explicable in terms of a master reaction (Thimann, 1935), but Went (1939), as a result of a careful analysis of the differential effects evoked by new synthetic hormones, brought forward strong arguments in favour of a multiple action of auxins. Whatever the final decisions on this point may be, it seems likely that some at least of the physiological properties of auxins are shared by coumarin, viz. reversible inhibitions of longitudinal root growth, stimulation of meristematic activity (rooting of cuttings and stimulation of lateral root production), cytological effects, etc. On the other hand, coumarin possesses properties apparently not possessed to any extent by auxins (i.e. inhibition of seed germination). Indeed some workers regard molecules of the coumarin type (i.e. unsaturated lactones) as acting complementary to the auxins, the delicate balance of growth stimulation (by auxins) and growth inhibition (by unsaturated lactones) constituting the ultimate chemical basis of growth control in plant cells (see Veldstra & Havinga, 1943–5; and Larsen, 1947). It is for future research to carry out an exact analysis of these various phenomena associated with coumarin action and it is hoped that such investigations may throw some light on the nature of the action of the auxins themselves.

SUMMARY

A series of experiments has been carried out on the conditions determining the extent and nature of the reversibility of growth inhibitions in plant roots (*Pisum sativum* and *Lepidium sativum*) induced by periods of exposure to low concentrations of 2:4-D. and coumarin. An apparatus is described for the accurate determination of root growth under carefully controlled conditions.

Results indicate that in low concentrations (0·1–0·33 p.p.m.) of 2:4-D. and with coumarin (30–100 p.p.m.) the initial rate of inhibition is limited by the rate of entry of inhibitor into the meristem cells. In higher concentrations of 2:4-D. (0·33–10 p.p.m.) it seems probable that this initial rate may be determined by the concentration of growth substance adsorbed at some colloidal interface, whence its action is exerted.

The primary inhibitions of longitudinal root growth induced by 2:4-D. and coumarin are, after a lag period of varying duration, completely reversible and this reversibility is independent of concentration over the range investigated (0·1–10 p.p.m. of 2:4-D. and 30–100 p.p.m. coumarin). The lag period, which varies with the inhibitor and the species, seems to be determined mainly by the restoration of the normal dynamic growth equilibrium and not by the speed of removal of the inhibitor.

It appears, however, that secondary irreversible inhibitions are attendant on the dislocation of the normal dynamic growth equilibrium and the possible accumulation of metabolites (e.g. amino-acids) to toxic concentration levels. Thus the total extent of growth recovery (indicated by the maximum growth rate reached after reversal) is determined mainly by the extent of these secondary inhibitions.

So-called maturation processes in the root meristem, indicated by the typical falling away of the growth rate after the grand period of growth, seem also to be delayed by 2:4-D. Coumarin in high concentrations, on the other hand, seems to accelerate these processes in the pea, resulting in a smaller degree of growth recovery after long inhibition periods.

Pea is rather more sensitive to coumarin than cress, whereas the reverse is the case with 2:4-D.

It is held that the term 'phytocidal', as descriptive of the primary growth-inhibiting actions of both growth substances and coumarin, is a misnomer. Instead the term 'phytostatic' is suggested as more accurately representing the completely reversible actions of these molecules.

REFERENCES

ALBAUM, H. G., KAISER, S. & NESTLER, H. A. (1937). The relation of hydrogen-ion concentration to the penetration of 3-indoleacetic acid into *Nitella* cells. *Amer. J. Bot.* **24**, 513.

AMLONG, H. U. (1936). Der Einfluss des Wuchsstoffes auf die Wanddehnbarkeit der *Vicia-Faba*-Wurzel. *Ber. dtsch. bot. Ges.* **54**, 271.

AUDUS, L. J. & QUASTEL, J. H. (1947*a*). Coumarin as a selective phytocidal agent. *Nature, Lond.*, **159**, 320.

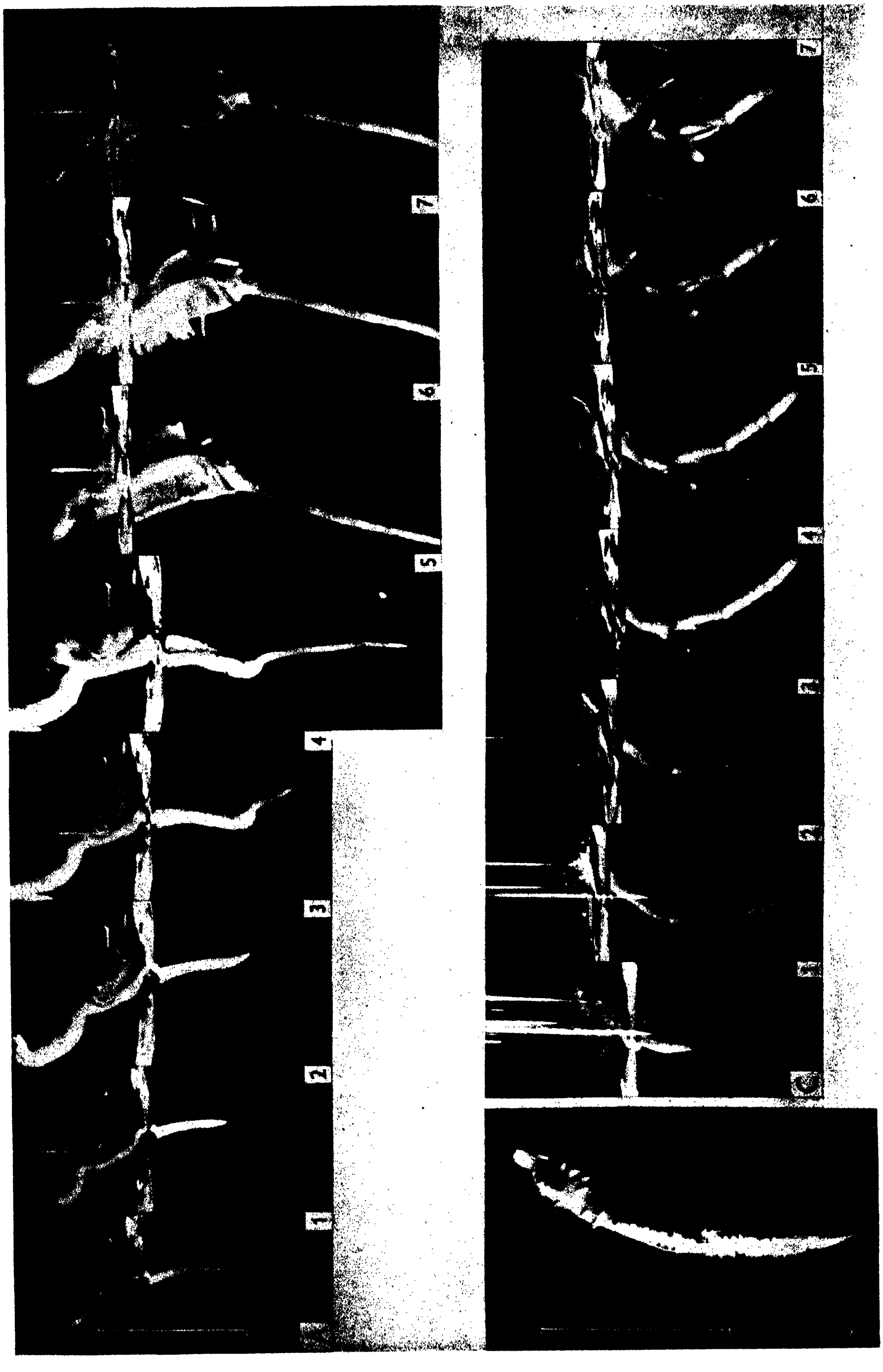
AUDUS, L. J. & QUASTEL, J. H. (1947*b*). Toxic effects of amino-acids and amines on seedling growth. *Nature, Lond.*, **160**, 222.

BORGSTRÖM, G. (1939*a*). Theoretical suggestions regarding the ethylene responses of plants and observations on the influence of apple-emanations. *K. fysiogr. Sällsk Lund Förh.* **9** (12), 1.

BORGSTRÖM, G. (1939*b*). Influence of growth promoting chemicals on the roots of *Allium*. *Bot. Notiser*, p. 207.

BOYSEN-JENSEN, P. (1936). Über die Verteilung des Wuchsstoffes in Keimstengeln und Wurzeln während der phototropischen und geotropischen Krümmung. *K. danske vidensk. Selsk. Biol. Med.* **13**, 1.

CORNMAN, I. (1946). Alteration of mitosis by coumarin and parasorbic acid. *Amer. J. Bot.* (Suppl.), **33**, 217.



AUDUS—PHYTOSTATIC ACTION OF 2:4-DICHLOROPHENOXYACETIC ACID AND COUMARIN

CORNMAN, I. (1947). The responses of onion and lily mitosis to coumarin and parasorbic acid. *J. Exp. Biol.* **23**, 292.

DELARGE, L. (1941). Étude de la croissance et de la ramification des racines en vitro. *Mém. Soc. Sci. Liège*, 2e sér. **5**.

FIEDLER, H. (1936). Entwicklungs- und reiz-physiologische Untersuchungen an Kulturen isolierter Wurzelspitzen. *Z. Bot.* **30**, 385.

GEIGER-HUBER, M. & BURLET, E. (1936). Über den hormonalen Einfluss der β-Indolylessigsaüre auf das Wachstum isolierter Wurzeln in keimfreier Organkultur. *Jb. wiss. Bot.* **84**, 233.

GRACE, N. H. (1937). Physiologic curve of response to phytohormones by seeds, growing plants, cuttings and lower plant forms. *Canad. J. Res. C*, **15**, 538.

LARSEN, P. (1947). *Avena* curvature produced by mixtures of growth promoting and growth retarding substances. *Amer. J. Bot.* **34** (7), 349.

LEVAN, A. (1939). Cytological phenomena connected with the root swelling caused by growth substances. *Hereditas, Lund*, **25**, 87.

MACHT, D. I. & GRUMBEIN, M. L. (1937). Influence of indole-acetic, indole-butyric and naphthalene acetic acids on roots of *Lupinus albus* seedlings. *Amer. J. Bot.* **24**, 457.

NEILSEN, N. (1930). Untersuchungen über einen neuen wachstumsregulierenden Stoff: Rhizopin. *Jb. wiss. Bot.* **73**, 25.

THIMANN, K. V. (1935). On an analysis of the activity of two growth promoting substances on plant tissue. *Proc. K. Akad. Wet. Amst.* **38**, 896.

THIMANN, K. V. (1936). Auxins and the growth of roots. *Amer. J. Bot.* **23**, 561.

THIMANN, K. V. & LANE, R. H. (1938). After-effects of the treatment of seed with auxin. *Amer. J. Bot.* **25**, 535.

THOMPSON, B. F. (1945). Tissue responses to physiologically active substances. *Bot. Rev.* **11**, 593.

VELDSTRA, H. (1944). Researches on plant growth substances, V. Relation between chemical structure and physiological activity. II. Contemplations on the place and mechanism of action of the growth substances. *Enzymologia*, **11**, 137.

VELDSTRA, H. & HAVINGA, E. (1943–45). On the physiological activity of unsaturated lactones. *Enzymologia*, **11**, 373.

WEILER, FR. (1938). Das Verhalten der Wurzeln unter der Einwirkung von Wuchsstoffen der *Avena*- und der *Zea*-Koleoptilspitzen. *Bull. Polonais litt. Sc. Nat.*, Ser. B, 1938.

WENT, F. W. (1939). Analysis and integration of various auxin effects. *Proc. K. Akad. Wet. Amst.* **42**, 581 and 731.

EXPLANATION OF PLATE

A. (1) Normal seedling of *Pisum sativum* 3 days after soaking and at the time of application of 10 p.p.m. 2:4-D. (2) Same seedling after 24 hr. exposure to 10 p.p.m. 2:4-D. and at the time of washing. (3)–(8) Same seedling after 1, 2, 3, 5, 6 and 7 days of washing.

B. 12-day-old main radicle of *P. sativum* after 8 days' continuous exposure to 1 p.p.m. 2:4-D.

C. (1) Normal seedling of *P. sativum* 4 days after soaking. (2)–(7) Same seedling after 2, 4, 6, 8, 10 and 12 days continuous exposure to 50 p.p.m. coumarin.

The vertical white lines are scales of 1 in., B and C being on the same scale. (For further explanation see text.)

VON POST'S WORK ON CLIMATIC RHYTHMS

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(With 6 figures in the text)

CONTENTS

	PAGE
1. Introduction	220
2. Theoretical background	221
(*a*) The hypothesis	221
(*b*) Climatic fluctuation	221
(*c*) Vegetational response	222
3. Discussion of evidence	222
(*a*) The historical record of rhythmic fluctuations in climate	222
(*b*) Apparently non-rhythmic changes	231
(*c*) Tentative conclusions	233
4. Suggestions for extended investigation	235
References	237

I. INTRODUCTION

It would not be unreasonable to hold the view that von Post has done more than any other scientist to give us an understanding of the post-glacial history of climate and vegetation. Ideas of climatic fluctuation were, of course, current before his time, and in the last thirty years several other investigators in this field have produced researches of the first importance in size and originality. Nevertheless, one might well base a claim for von Post's pre-eminence on the single fact of his introduction of the statistical method of pollen analysis in the second decade of this century. Over and above this, however, we owe an immense debt to his subsequent work, as can perhaps best be judged from the translation (*New Phytol.* 1946) of a recent paper by von Post (1944) which has given English readers a 'world view' of the results of thirty years of research in pollen statistics. From it we may gather something of what he has himself contributed both in data and ideas.

These ideas form an important part of what we may call 'historical ecology', and, indeed, ecologists in this century have been fully alive to the value of the historical approach in the study of vegetation. Not only do we think always in terms of the dynamic ideas that derive from the successional doctrines of Clements, but we have come increasingly to recognize the profound effects that man has exerted on vegetation at any time during the post-glacial period when the population of a district has reached a sufficient density. Again, in dealing with coastal districts, we are always ready to make allowance for the possible influences of past changes in the relative levels of land and sea.

If these aspects are ignored in the present paper, it is not because I do not recognize their importance, but because I want to deal as briefly as possible with some ideas within the field of climatic change, ideas arising from the acceptance of von Post's suggestions and the attempt to amplify them a little. In so doing, it may be possible to bring into a single perspective a number of ideas which have been discussed by others besides von Post. Moreover, since at present a number of young students are becoming interested in the problems of historical ecology, it may be useful to state explicitly certain points that arise in the interpretation of pollen diagrams, and to suggest lines of practical approach, some of them dealing with problems of detail, limited in scope, and therefore simple for a beginner to handle.

There is no need to recapitulate the substance of von Post's paper. For the present purpose, the outstanding features to bear in mind are: first, the idea of an upswing of climatic conditions to an optimum after the last glaciation, with subsequent deterioration; secondly, the detailed analysis of pollen curves and of peat stratigraphy to reveal the story of small-scale fluctuations; and thirdly, the idea that it may be possible to interpret climatic fluctuations in general in terms of various rhythmic oscillations. In the essay which follows, I shall adopt this last idea, which is expressed by von Post in a most scientifically cautious way, and shall state it in the form of a broadly generalized hypothesis. This is done not in the belief that such a generalization can now be derived from the existing evidence, nor even with the presumption that it will one day be proved correct, but because it seems to offer a basis for suggesting ways of accumulating useful data which might not otherwise be sought. In discussing the evidence, I shall put forward interpretations in order to illustrate the possibilities of the hypothesis. Many of these interpretations are open to varied attack, and I would emphasize their speculative character and illustrative purpose.

2. THEORETICAL BACKGROUND

(*a*) *The hypothesis*

The three main points which appear to be involved in von Post's idea are as follows:

(1) Fluctuations of climate may be analysed into a number of rhythmic variations of one or more master factors in the climatic complex. For simplicity, each rhythm will be considered as having simple harmonic form.

(2) These rhythms will be reflected in one way or another, at one place or another, in historical records of vegetation, of animal population (including man), or of geological phenomena.

(3) Where the historical record reflects a change which is apparently sudden and steplike, this will be explained by a more detailed consideration of the record.

Before discussing the hypothesis in relation to the existing data, it is necessary to give some explanation and amplification from the theoretical aspect, in regard both to climate and to vegetational response. Most of these points are discussed explicitly, though of course in different terms, or else are implicit, in von Post's paper.

(*b*) *Climatic fluctuation*

(i) Since climate is determined by so many interacting factors, and is not itself to be described in simple quantitative terms, the phrase 'climatic fluctuation' has only the vaguest significance. One is bound to postulate, as a first approach, that if the character of a climate exhibits some periodicity in its fluctuations, this is brought about by the variation of some one measurable factor, the master factor, which is, from our point of view, an independent variable. If there are apparently two rhythms involved in the climate as a whole, this might be due either to a single master factor having a complex variability which can be analysed into two simple rhythms, or to two different master factors, each with a rhythmic variation, which imposes itself on the resultant climate.

(ii) If two or more rhythms of different periodicities are compounded into a single curve of variation, a coincidence of maxima or minima may give a sudden large deviation from the mean, when the amplitudes of the different rhythms are not too widely different.

(iii) It seems likely that many of the physical patterns which determine the climate of a region have a certain stability. Thus, for example, the course and character of ocean currents, or the normal paths of movement of cyclonic centres, are features of the earth's surface which are determined, for any given configuration of the solid crust, by the radiant energy incident on the earth's surface. Yet it is possible that these might remain stable over a definite range of incident energy, so that they would only be noticeably altered when the incident energy passed beyond some threshold value. The alteration, if it came, might cause a profound and sudden climatic alteration in some or all parts of the earth, and yet the variation of the master factor might all the while have been following the smooth gradual course of a harmonic curve.

(c) *Vegetational response*

(i) The idea of a threshold value for change, which has just been expressed in relation to the climatic complex, applies with equal or greater force in a consideration of vegetation types. Thus any particular type will have a range of tolerance in regard to climate, and will not change unless the climate changes beyond the threshold of tolerance. Hence any particular alteration of the local resultant climate (not to mention an alteration of some master factor) need not be expressed at all by the vegetation of that locality. It is implied in paragraph 2 of the statement of the hypothesis, that any climatic change will cross *some* threshold, but it does not tell us where to look for the phenomena that will demonstrate the crossing. It is in relation to this point that there is, perhaps, the greatest scope for liveliness of mind in thinking of new places to search.

(ii) Even supposing that a threshold has been crossed, and a new type of vegetation has taken the place of the old, there will be a time lag in the expression of the climatic change owing to the natural processes by which plant communities become established. In other words, just as there is no instantaneous establishment of climax vegetation in a primary succession under constant climate, so there is no instantaneous attainment of equilibrium in response to a changing climate.

3. DISCUSSION OF EVIDENCE

(a) *The historical record of rhythmic fluctuations in climate*

(i) The multiple glaciations of the Pleistocene ice-age form the clearest and best known example of large-scale climatic oscillations. There are certain difficulties, probably not insuperable, in reading the data as the record of a simple rhythm. We may leave on one side the difficulties of geological interpretation, and the necessarily tentative nature of correlations between the glacial phases of widely separated ice-sheets, and assume for the present purpose that Flint (1947) is correct when he says that 'according to the present weight of evidence the great climatic fluctuations were simultaneous throughout the world'. We still have to account for the evidence that the central interglacial period (the Mindel/Riss of the Alps and the Yarmouth of North America) was of much longer duration than the preceding or the succeeding interglacials.

This receives an ingenious explanation on Simpson's theory of the ice-age, which postulates, not four cycles in the rhythm of the master factor, inducing four glaciations, but two only. Simpson's theory is fascinating to the non-geologist, who cannot bring geological criticism to bear upon it, but its very fascination gives rise to the feeling that it is 'too good to be true'. Quite apart from anything else, it would be wrecked by the discovery that there had really been five (or more) major glacial periods instead of four—the number which at present seems to be the maximum recognizable in the northern hemisphere. Flint has stressed that the possibility of such a discovery must always be kept in mind. His own theory* resembles that of Simpson in that it is based on the idea of fluctuations in solar radiation; in other respects it is widely different. It has the advantage that it would not be affected by the number of major glacial maxima which are recognized, and moreover it would make it possible to hold that periodic fluctuations in intensity might be a permanent feature of the sun's radiation, at any rate within the time limits of the earth's known geological history. For, on his theory, an ice-age only results from the *combination* of orogeny with fluctuating radiation. If the latter were the sole causative agent, then the Pleistocene period (as also the Permian and other glacial periods) must represent an occasional 'aberration' of the sun's physical state—supposing, of course, that we eventually prove correct in assuming that relatively large-scale changes in solar radiation may actually occur. It is not relevant to my purpose, even if I were competent, to discuss this idea or other ideas such as that of Milankowitch, of which the weaknesses have been summarized by Flint. Still less am I accepting any theory of the cause of the ice-age, or making any suggestions as to the nature of the master-factor which is concerned in any of the other hypothetical rhythms which are discussed. On the contrary, it is essential that the historical record should be thoroughly studied first in order to see whether there are in fact any rhythms for which explanations must be sought.

Returning to the problem of the uneven lengths of the interglacial periods, it should be noted that there is considerable variation in the estimates of the discrepancies. Thus, while on Penck's estimate the ratio of the length of the second interglacial to the length of the third interglacial has the value 4:1, the ratio according to the estimates discussed by Flint would not be greater than 2:1. The length of an interglacial is in any case a less well-defined concept than that of the time interval between successive maxima or minima, and estimates of it will vary both with the locality and with the basis of estimation.

In view of these uncertainties, I shall simply regard the ice-age as exhibiting four cycles within a period of at least a million years. In other words, it exhibits a cyclic fluctuation with a period of the order of 250,000 years.

One may find various estimates of the time elapsed since the last glacial maximum. The longest I have met is that of 55,000 years quoted by Flint for the North American continent. Even this implies that we are still, at the present day, less than a quarter of a cycle away from the maximum. If this conclusion is correct—and it seems to be generally held—it is of considerable importance, for it means that the whole of our 'post-glacial' history, however widely or narrowly we interpret that phrase, must be viewed against the background of a 'major drift' of climate away from the conditions which give glacial maxima. This idea may be represented schematically as in Fig. 1. The segment *AB* of the main curve then represents the trend of general amelioration which we must keep in mind in our interpretations of minor fluctuations.

(ii) The margins of the ice-sheets have not shown a steady retreat from the positions of maximum extent. On the contrary, there is abundant evidence of temporary readvances of the ice, which interrupted the general course of retreat. The situation in North America may be summarized by a quotation from Flint (1947) which has a special interest for the present discussion. Flint writes: '...in central North America four glacial stages within the Wisconsin stage are recognized. The first of these marks the maximum extent of the ice-sheet. The second, third, and fourth mark re-expansions of the ice-sheet after it had shrunk by an unknown though probably not large amount. According to the best time estimates the ice-sheet uncovered the first or Iowan drift 55,000 years ago, whereas the third or Mankato re-expansion of the ice ended about 25,000 years ago.' Thus we have three readvances (the Tazewell, Cary, and Mankato stages of the Mississippi basin) in 30,000 years, and we could therefore interpret them as expressions of a rhythmic fluctuation with a periodicity of the order of 10,000 years.

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* Since Flint's statement of his hypothesis appeared quite recently, it may not be familiar to British readers. He calls it the 'solar-topographic hypothesis'; it suggests that the sun's radiant energy fluctuates about a mean, and that the minima of the radiation curve will lower the earth's temperature to an extent sufficient to produce ice-sheets in temperate regions, provided that there are mountain ranges of sufficient height to act as centres of snow accumulation and hence glacier-formation. It follows that ice-ages will occur towards the end of, and immediately after, periods of intensive orogeny, and Flint ascribes the Pleistocene ice-age to the widespread orogenic movements of the Miocene, Pliocene, and early Pleistocene periods.

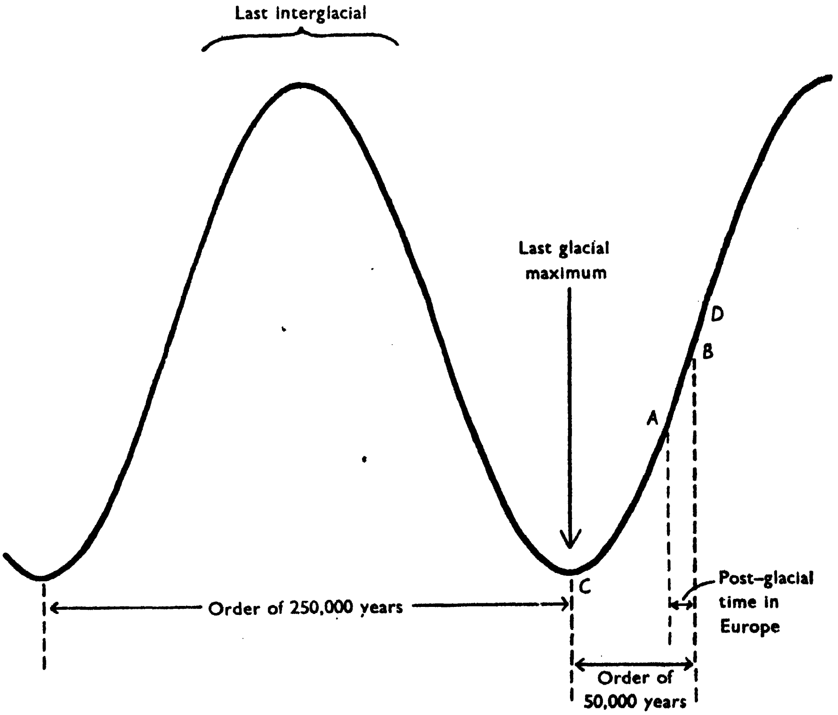

Fig. 1. Schematic representation of the most recent major climatic fluctuations.

Turning to the European evidence we may consider the Alpine 'Rückzugsstadien' of Penck, named by him the Bühlstadium, the Geschnitzstadium and the Daunstadium. Wright (1937) discusses the dating of these, and states: 'Thus we have roughly, since the Daunstadium, 7000 years, since the Bühlstadium, 20,000 years.' Wright gives 20,000 years as being the mean of two separate estimates of 16,000 and 24,000 respectively. Since the Geschnitzstadium comes in between, we have the result that three readvances have occurred in a period of about 20,000 years. These readvances clearly could not be correlated with the American Tazewell, Cary, and Mankato stages, even if transatlantic correlations should ever be satisfactorily achieved, since the Mankato, the youngest of the three, is dated back as far as 25,000 years ago. Nevertheless, the estimates quoted for the two sets fall sufficiently closely to the same order of magnitude to raise at least the thought that all the six readvance stages might be expressions of one rhythmic series.

Bearing in mind the 'major drift' discussed above, we may well ask whether the same rhythm may not have left its impress during late-glacial and post-glacial phases, especially in the better-known records in Europe. That is to say, can we recognize any 'climatic retrogression', expressed in Europe in some way other than by advances of the ice-sheet which might seem, by its timing, to be a member of the same series? The obvious suggestion is that von Post's climatic deterioration is the phenomenon that we are seeking.

In making this suggestion there are at least two difficulties to be faced. The first is that of assigning any date for the maximum of the deterioration. The present evidence goes to show that it set in gradually, and the position of the maximum presumably lies later than 500 B.C., the approximate date of Weber's Grenzhorizont. It is not easy to decide whether the maximum has already been passed at the present day, because of the profound modifications of the historical record which have been brought about by human activities during the last thousand years.

It is therefore of especial interest to consider the important conclusions which have been drawn by Cooper (1942) from his observations on the glacial fronts in Alaska. He finds that they have advanced considerably since the period of post-glacial warmth maximum, and have been in a position of maximum extension during the last 200 years. Although Cooper cites localities where glacial recession is in progress to-day, it does not seem necessarily certain that we are past the maximum of this particular climatic retrogression: at any rate, one can hardly help accepting Cooper's inference that the Alaska phenomena form an expression of the same climatic fluctuation which von Post describes for Europe.

In attributing von Post's fluctuation to this hypothetical rhythm with a periodicity of the order of 10,000 years, we meet a second difficulty, namely, that the idea conflicts with von Post's own tentative suggestion that the post-glacial climatic optimum represents the whole of an interglacial period. If, however, the schematic representation shown in Fig. 1 comes anywhere near the truth, such a suggestion cannot be accepted, and we must surely regard the recent retrogression as a lesser fluctuation imposed on the major drift. Such a view would not be incompatible with the data given in the diagrams (von Post, 1946, Fig. 9) of interglacial and post-glacial deposits in Denmark, which von Post uses in support of his suggestion.

Difficulties arise again when we go back from the von Post deterioration to the preceding oscillation of the series we are postulating, namely, that which produced the Daun stage in the Alps. It would seem that the correlation and dating of the geological and botanical histories in the Alps are not yet securely established, though Welten's varved series in the Faulenseemoos gives a time scale since about 8000 B.C. Hence the correlation of Swiss events with those of Scandinavia and Britain must be even more tentative. It would be most desirable to know what phase in north-west Europe could be correlated with the Daun readvance; could we, for instance, equate it with the post-Allerød retrogression? There is, first of all, a difficulty as to the date of the Daun stage. Wright gave 5000 B.C. as the latest date, but it might well have been earlier. On the other hand, if we consider the snow-line it is said to have represented (300 m. below the present), the altitude of the Faulensee

(600 m.), and the date which Welten gives for the freeing of the Faulensee area from ice (7700 B.C.), it is hard to push the date back very far. If so, it would be hard to correlate it with the post-Allerød retrogression which appears to be dated not later than 8000 B.C.

Also, the evidence concerning this latter phase suggests that it did not last for more than 300–500 years, according to the discussion given by Pennington (1947). In this case it could hardly be considered to be a phenomenon on the same scale as the von Post deterioration. Again, we may quote a point from Godwin (1947), who refers to 'the apparent recognition of a double character of the allerød warm period, or, perhaps more probably, in an earlier and less pronounced interstadial than that now spoken of as the allerød'.

A reading of the data and discussions given, for example, by Lüdi (1944), Welten (1944), Härri (1940), Iversen (1947), and Pennington (1947), reinforces the impression that during this still obscure late glacial period there was more than one 'Klimarückschlag'. If this is so, was not the Daun merely one of these, and so also not comparable with the von Post deterioration?

In the face of all these doubts we may either suppose that the suggested 10,000-year rhythm is entirely mythical—a result quite likely to emerge from our eventual knowledge of the facts—or else we may very tentatively suggest that the whole of the period between 10,000 B.C. and 6000 B.C. included the maximum of a phase of retrogression homologous with the von Post deterioration, and that lesser oscillations were also recorded during that period.

(iii) The preceding paragraph has introduced the idea of a lesser fluctuation superposed on the postulated 10,000-year rhythm. The same idea is illustrated, and documented with much more definite evidence, by the recurrence horizons of Granlund which are superposed on the von Post deterioration.

In earlier days the study of lowland raised bogs gave great prominence to the widespread occurrence of Weber's Grenzhorizont, which was taken to indicate a sudden onset of the 'sub-atlantic climate'. Owing to the work of Granlund (1932) it has appeared that Weber's horizon is one of several of similar type, and it seems that this one was especially prominent in just that kind of bog which was mainly investigated in the early part of this century. Granlund recognized five main recurrence horizons (rekurrensytor, or RY), of which the central one was equivalent to Weber's Grenzhorizont: Granlund has dated his five horizons by archaeological correlations as follows: RY V, 2300 B.C.; RY IV, 1200 B.C.; RY III, 500 B.C.; RY II, A.D. 400; RY I, A.D. 1200. The intervals are not even, but archaeological dating cannot yet be considered as highly exact, and it is not hard to conceive these horizons as expressing a minor climatic rhythm with a periodicity of the order of 900 years.

We owe to Godwin (1946) a very considerable clarification of our ideas concerning British ombrogenous bogs and the types of response which they have shown to what I will call for convenience the 'Granlund rhythm'. He has shown that different members of the Granlund series may form the 'major' boundary horizon in different bogs; by 'major', I mean that horizon which indicates the most marked change from a vegetation type characteristic of rather drier conditions to a type characteristic of a much wetter climate. Thus, whereas in Tregaron bog (Godwin & Mitchell, 1938), the 'major' boundary horizon is Granlund's RY III, in certain Irish bogs, and in Ringinglow bog in the Pennines, the main change is associated with RY IV. More than this, Clapham & Godwin (1948) have shown by their work in Somerset that even in bogs which originally appeared to be 'orthodox' in their stratigraphy, the supposed boundary horizon may really be a double structure. Their results indicate that while in places there is a stratigraphical record of RY IV, the majority of the profiles show strongly marked effects first at RY III and then again, sometimes even more clearly, at RY II.

It is worth considering a few examples of British bog stratigraphy from this point of view, because they form a striking illustration of the idea of threshold values in the relation between climatic change and vegetation type. Fig. 2 presents the idea schematically. The ordinates represent the hypothetical magnitude of whatever climatic factor it is that favours more rapid and abundant growth of *Sphagnum* species on the bog surface; we may for convenience call it the 'moisture factor'. Perhaps the rainfall/evaporation ratio is the most plausible variable to suggest, but for the present argument there is no need to decide on the precise nature of the moisture factor. The horizontal lines represent the threshold values of the moisture factor which separate the graded series of vegetation types indicated on the left of the diagram. It is postulated that any given stage in the course of the climatic fluctuations over a large area will give a value of the moisture factor which will vary from locality to locality according to the altitude, the general situation, and the topographic characteristics of each locality. Thus the moisture-factor curves for different localities should be roughly parallel in behaviour but not necessarily coincident. Further, unless a given climatic oscillation causes the curve for the moisture factor at a given locality to cross a threshold line, then that climatic oscillation will not be expressed by the vegetation at that locality.

On this basis the diagram attempts to bring some unity into the somewhat divergent stratigraphic records in the four following bog areas:

(1) *Shropshire* (Hardy, 1939). In the bogs described by Hardy, ombrogenous peat did not succeed topogenous peat until well into zone VII (of Godwin's zonation scheme), probably after the beginning of VII*b*. The stratigraphy gave evidence of four recurrence horizons, the first two expressed by pine growth on the peat (horizons P2 and P3 of Hardy's terminology), the later (Gr 1 and Gr 2) being recurrence surfaces of the Granlund type. Gr 1 is identified with the Grenzhorizont, or Granlund's RY III, and I shall venture to take the other three horizons as also representing members of the Granlund series.

(2) *Tregaron* (Godwin & Mitchell, 1938). Here there are only two recurrence layers, the earlier (Gr), recognized by the authors as the Grenzhorizont (RY III), and the later (Ret.), called by them the 'retardation layer', which may reasonably be correlated with RY II.

(3) *Ringinglow* (Conway, 1947). Here the ombrogenous peat began to form at the B.A.T., and the Ulm. horizon falls at a marked alteration of the peat texture. RY V is not at present recognized, but RY IV seems to have been the episode leaving the most marked record; it was described as the '*Menyanthes* horizon' (Men.). RY III is only vaguely expressed in the stratigraphy, and after this time the moisture conditions at this altitude (1350 ft. or 400 m.) would seem to have been always well above the threshold for the most rapid type of sphagnum peat accumulation.

(4) *Kinder Scout* (Conway, unpublished). Here the lower layers repeat the Ringinglow story. The data concerning the Granlund series are not yet fully clarified, but it is likely that RY IV will be consistently recognized in these mountain-top profiles, from both

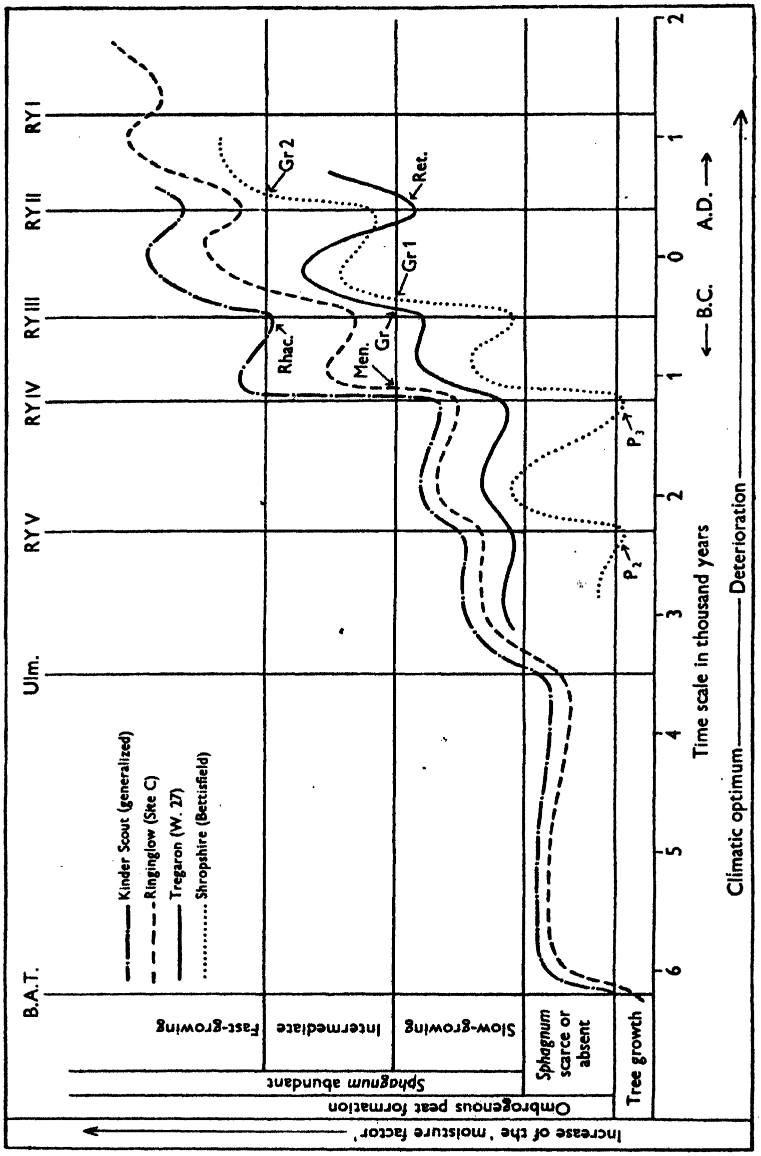

Fig. 2. Schematic interpretation of the stratigraphy of four British peat profiles in terms of a hypothetical 'moisture factor'.

Kinder Scout and Bleaklow, at altitudes of about 2000 ft. (600 m.). A frequently recognized layer of *Rhacomitrium lanuginosum* is found at about the level where RY III could be expected. It is hoped shortly to date this horizon definitely in pollen-analytical terms, but in the meanwhile the *Rhacomitrium* layer (Rhac.) is assumed equivalent to RY III. Later recurrence horizons may well be recognizable in these profiles when they are fully investigated, but they cannot at present be indicated in Fig. 2.

The curves of Fig. 2 have been so drawn that they shall express all these points just described. It may be seen that the resulting diagram can also be taken as an expression of the general drift of the von Post deterioration since zone VII*a*, with minor fluctuations (the Granlund series) superposed upon it.

(iv) Let us lastly consider some scattered pieces of evidence concerning rhythms of still smaller periodicity.

(*a*) There is first the kind of data contributed by von Post and others on the 'microstratigraphy' of Scandinavian bogs and the associated pollen-curve variations. An illustration is given in figure 11 of von Post's paper (von Post, 1946). Here we see a fact that I have ignored so far in dealing with the Granlund series—namely, that there are other recurrence horizons in between them. The five Granlund horizons are then only the major members of a more numerous series, the minor members of which have not so far been recognized over any very wide area. We see also how various pollen curves show fluctuations which can be correlated with the recurrence horizons, and the way in which von Post has effected an analysis of the birch pollen curves into rhythmic fluctuations of different periods and amplitudes, and an accompanying 'general drift'. The smallest periodicity exhibited in this analysis is of the order of 250 years.

(*b*) Observations of quite a different sort on the bogs of Minnesota, U.S.A., have made a strong impression of the possible effects of short-term climatic fluctuation. This impression arises mainly from the very sharp interzonal boundaries that are almost always found between the seral stages in the hydroseres that are found in such numbers in that area. There was every indication that the succession proceeded not by a slow steady advance, but by phases of relatively rapid advance separated by phases of little or no advance. Fig. 3 illustrates this by means of a narrow belt transect across a typical example. The transect was two paces wide and passed from the upland bog margin at the left to the water margin at the right. Not only is there a sharp line separating the bog-forest from the younger floating sedge-mat zone, but the bog-forest itself shows clearly demarcated zones, each of which is more or less homogeneous in character.

Another feature of the Minnesota bogs at the present day is the frequent occurrence of bogs in which a *Chamaedaphne-Sphagnum* association extends right to the water margin. Such an association does not itself colonize open water, but its invasion into a normal pioneer-mat is much favoured by a lowering of the water table. It is, therefore, plausible to suggest that these marginal *Chamaedaphne-Sphagnum* associations represent the effects of the dry period which was so marked a feature of the nineteen-thirties in central U.S.A. If we look now at the precipitation data for Minnesota, given in Fig. 4, we see that the 'dry thirties' are not an isolated occurrence, but form the culmination of a long downward trend of average level, which began about 1905. In fact, the general appearance of Fig. 4 makes it strongly tempting to see in it part of a harmonic variation with a period of about 60 years. The temptation has, of course, to be resisted, since the appearance could be purely fortuitous. Nevertheless, it seems that it would be worth

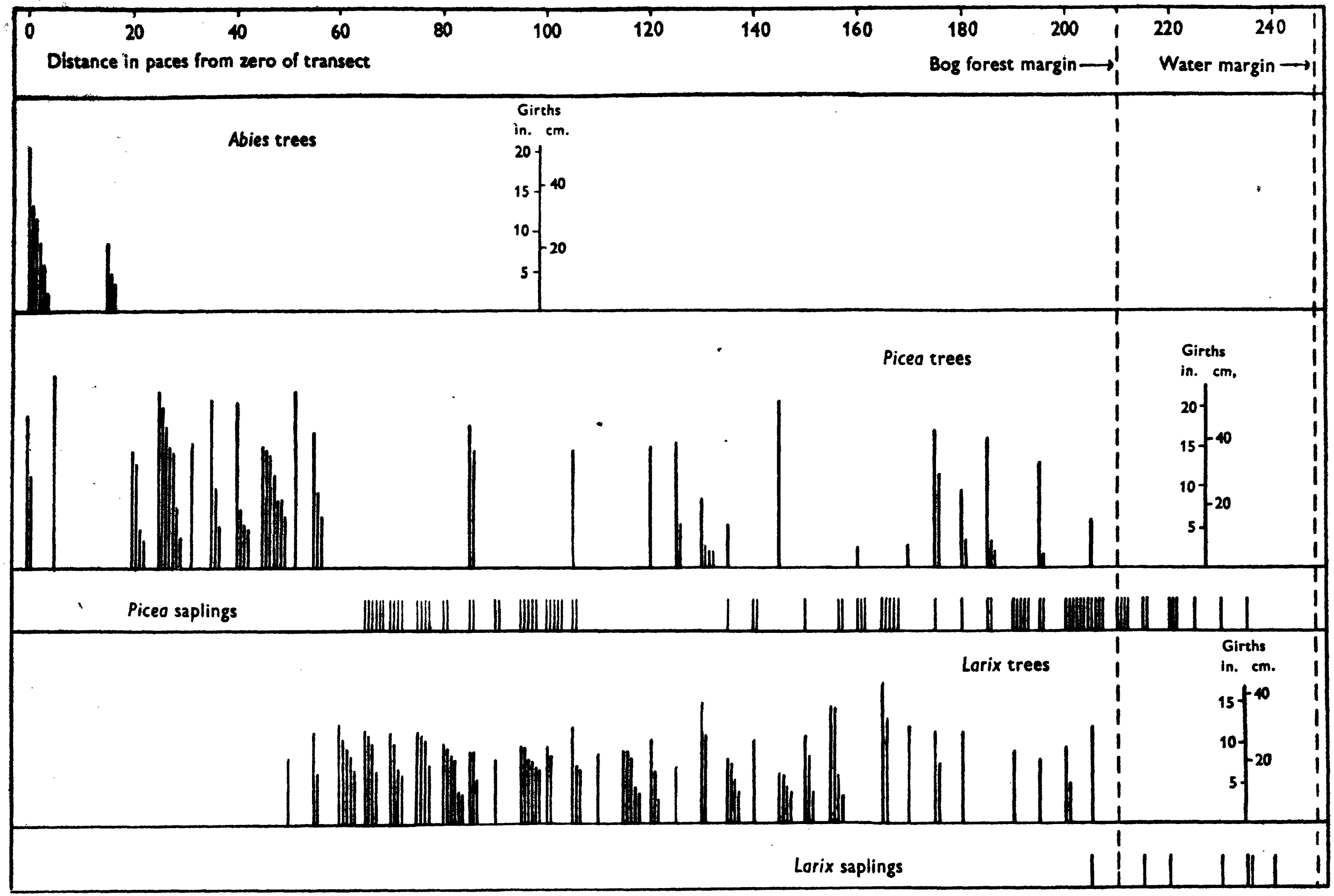

Fig. 3. Transect across the zones of vegetation on a bog in Minnesota, U.S.A. The girths of trees and number of saplings were recorded for each strip of five paces along the transect and two paces in width.

while finding out whether the vegetational records of bogs in this area confirm the suggestion of spasmodic development and, if so, what length of period may be associated with the climatic fluctuations which presumably determine the course of development.

(*c*) The regeneration complex vegetation of raised bogs has been generally regarded as representing a cyclic type of development which will proceed under stable climatic conditions, if these are of the right sort. Without offering any definite suggestion, one might perhaps at least put forward the question as to whether we should have to alter our interpretation of the observed vegetational mosaic which we call the regeneration complex, if it should be proved that climates are never stable but always in some phase of a larger or smaller oscillation? A relevant paragraph may be quoted without further comment from the account of Tregaron bog given by Godwin & Conway (1939): 'It is interesting...to recall the comments made in describing the Regeneration Complex to the effect that stages of pool invasion were very frequent and degenerate tussocks very infrequent. This departure from expectation is not remarkable if the Regeneration Complex is only just re-establishing itself over an older drier surface. We feel that possibly similar anomalies, not only in Tregaron, but in other West European bogs, could be resolved by recognizing the importance of recent climatic fluctuation.'

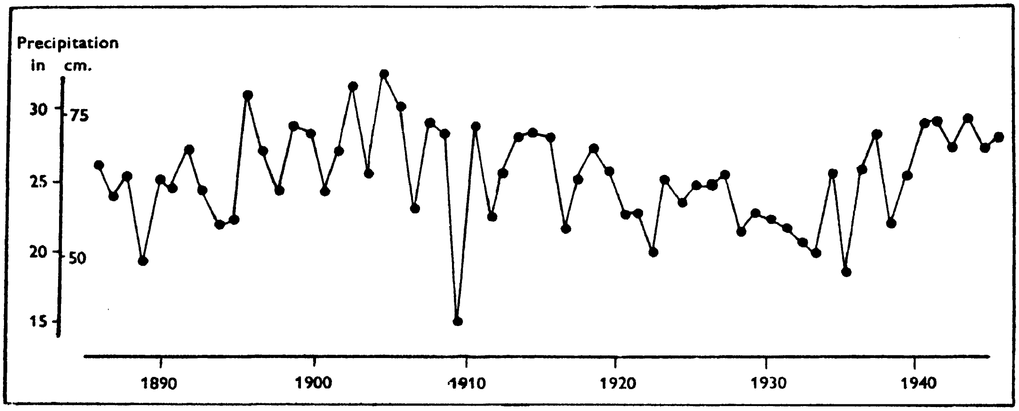

Fig. 4. Mean annual rainfall data for the state of Minnesota, U.S.A., since 1886. (*Rep. U.S. Dept. Agric. Weath. Bur.*)

(*b*) *Apparently non-rhythmic changes*

In the records of post-glacial vegetation there are certain horizons which seem to indicate climatic changes of a type which is not repeated, and which cannot therefore be directly fitted into the rhythmic hypothesis. The most important of these is the so-called Boreal-Atlantic Transition (B.A.T.). Whatever may have been the exact nature of the climatic change, it was clearly very definite. It sufficed, for example, to initiate the formation of ombrogenous peat on the upland plateaus of the southern Pennines where previously no peat was formed. An examination of English pollen diagrams suggests also that the change took place in a relatively short space of time. Consider, for example, the *Alnus* pollen curve, whose sudden rise to replace *Pinus* is the diagnostic feature of the B.A.T. in British diagrams; the rise is, so to speak, explosive, and contrasts with the gradual entry that usually typifies the curves for oak, elm, and lime. This point emerges very clearly from the data obtained by Welten (1944) from the Faulenseemoos near the Lake of Thun. The bottom deposits from which his pollen diagrams were obtained showed annual laminations which allowed very close dating of the phases of the diagrams, and gave for the first time a picture of the rates of attainment of relative equilibrium by the vegetation.

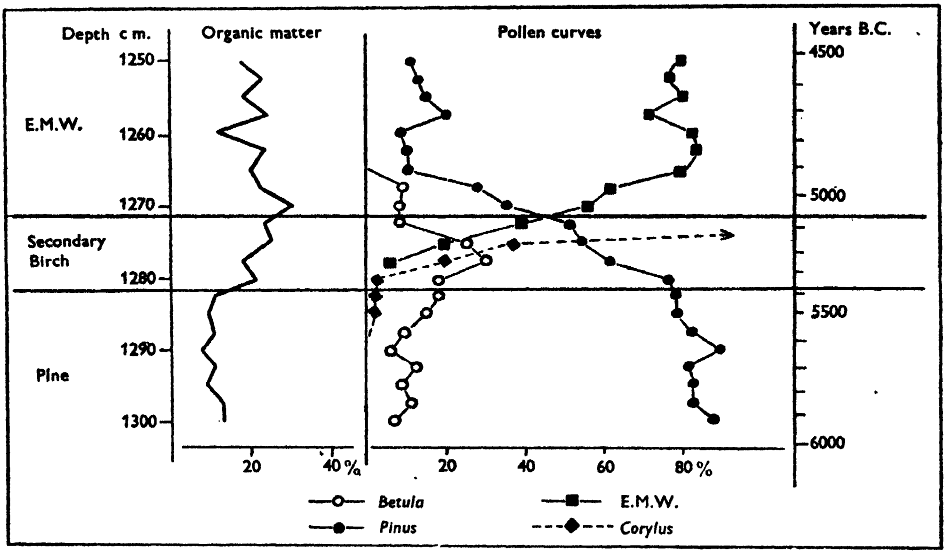

Fig. 5. Enlarged redrawing of part of Welten's diagram for profile XI of the Faulenseemoos. (Welten, 1944, fig. 15.)

Fig. 5 reproduces a small part of figure 15 in Welten's paper. It shows the transition from Welten's pine phase below, to E.M.W. at the top, with an intervening phase which he calls 'Secondary Birch'. This latter covers a period of 350 years approximately. It seems clear from this and other of Welten's diagrams, and from his discussion of correlations, that this transition is the same as that which we see in north-west Europe, from a coniferous forest to a hardwood forest; in other words, it must be the equivalent of our B.A.T. If we now examine the actual curves closely, it will appear that the change from the steady values of the pine phase to the steady values of the E.M.W. covers something like 500 years. On the other hand, it is obvious that the change sets in immediately after the lower horizon of the secondary birch phase, i.e. at 1281 cm. depth, or about 5380 B.C. Moreover, at this horizon the curve for the organic matter percentage shows a rise between one sample and the next above, which brings the values immediately to the average level for the subsequent E.M.W. period. The pollen curves also begin to change immediately, and their behaviour clearly reflects a 'successional' story. Thus, as the pine diminished owing to the new type of climate, the rapidly spreading birch (the species was probably *Betula verrucosa*) colonized the opening forest. It gave way itself, however, under the competition with the more slowly developing E.M.W. and *Corylus*. The 'Secondary Birch' phase on this interpretation would not be a true phase, or zone, comparable to the E.M.W. phase, but an expression of the time lag between the climatic change and the establishment of a new vegetation-climate equilibrium—the period of adjustment to the relatively sudden climatic change whose main incidence may be seen at a horizon, rather than through a phase.

If this interpretation is accepted, it would mean that the major climatic switch took place inside a hundred years. How then can we suppose that we are dealing with a smooth harmonic type of variation such as we must postulate on the rhythmic hypothesis? Two considerations are relevant here.

(1) The change in the vegetation everywhere in north-west Europe is from a conifer-dominated forest to a forest of deciduous trees. Does this not rather strongly suggest that whatever may have been the full character of the climatic change, it did involve an alteration from a climate with precipitation mainly in the form of snow, to one with precipitation mainly in the form of rain?

(2) Recent work by Godwin (1945) has shown that the period immediately preceding the B.A.T. was the time of maximum rate of eustatic rise of sea-level around the British coastline, and those coasts of Europe adjacent to Britain. This must imply a rapid rate of recession of the ice-fronts. Moreover, the very great increase of the area of sea compared to land must in itself affect the climate of maritime territories.

Could not these points receive at any rate a preliminary interpretation from the idea that the B.A.T. represents the point at which the major drift of post-glacial climatic improvement reached a critical value in relation to ice-formation? There would then be no need to postulate a sudden step-like change in a master factor, which we may think of rather as varying smoothly along the long-period, large-amplitude rhythm of the main glaciations. But when it passes over that most critical threshold value which determines rain rather than snow as the characteristic form of precipitation, we can see what profound consequences must ensue. The difference in, shall we say, mean annual temperature may have been very slight as between the late Boreal and the early Atlantic periods, yet the diminution of snow, the lack of nourishment of glaciers, and the increase in sea area, may all have combined together to produce one of the steepest gradients in climatic change for which we have any evidence.*

If, then, it should prove possible to explain the B.A.T. in terms of the rhythmic hypothesis, there would be good hope that some of the other apparently sudden changes might have similar explanations.

(*c*) *Tentative conclusions*

In general, it appears that the longer the period, the larger the amplitude of the fluctuations of the master factors, as judged by the nature of the effects shown by the geological and botanical records. A long-period fluctuation with small amplitude would be hard to detect in the historical record, and the absence of large-amplitude but short-period rhythms accounts for our common-sense acceptance of climate as a more or less constant feature of our environment.

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* One conclusion that could logically be drawn if such an interpretation were correct, is that the phenomena which we associate with the B.A.T. need not necessarily have occurred simultaneously all over Europe. For the major drift in the master factor might well cause local climates to cross the critical threshold at slightly different times, owing to difference of latitude and so on. This would mean that, though we should expect broad agreement, we need not expect to find that a dating derived, say, by Welten at the Faulensee would agree within a hundred or even a thousand years, with one deduced, say, in Scotland.

The curve shown in Fig. 6 represents roughly the curve obtained by compounding the three largest of the rhythms about which I have been speculating. It includes, that is to say, the 'major drift', the medium-scale oscillations of the glacial readvances, and the lesser rhythm of the main Granlund series. The rhythms of smaller periodicity are not included. The periods of these three are taken in the ratio 250:10:0·8; Fig. 6A shows the curve for one-quarter of the period of the largest rhythm, and corresponds to the section *CD* of Fig. 1. Fig. 6B shows a part of 6A enlarged to clarify some of the details.

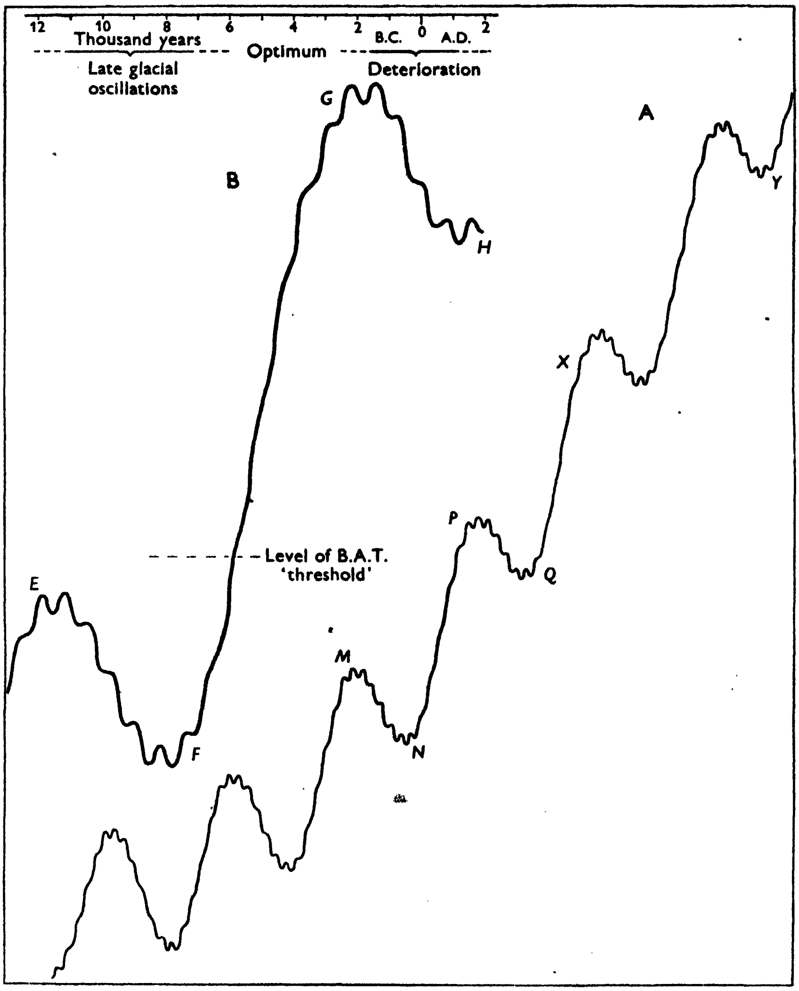

Fig. 6. A, resultant curve of three harmonic variations with periods in the ratio 250:10:0·8, and amplitudes in the ratio 180:15:1; B, Section *XY* of the curve A, enlarged to illustrate some of the speculations discussed in the text.

The choice of relative amplitudes in this diagram is of course arbitrary, and merely represents pictorially a subjective interpretation of the existing data. If the curve as a whole bears any relation at all to the course of climatic variation it suggests that, in the case of these three rhythms, the superposition of maxima and minima is unlikely to have caused any very prominent effects. This conclusion could not apply to the smaller rhythms, not included in Fig. 6, which von Post has suggested on the basis of the *Betula* curves; these would seem to be sufficiently similar in their order of magnitude to give occasional resultant displacements far from the mean values.

Perhaps the most useful conclusion to be drawn from Fig. 6 is that the effect of a maximum or minimum in a minor rhythm will depend on whereabouts it falls in relation to the phase of a major rhythm. This statement may be taken in two ways, both of which are relevant.

In the first place, if we consider segments *MN* and *PQ* of the curve, with a general similarity of shape, their expression in the vegetational record will be different because their effects are set at different climatic levels; we may see here, for example, why the von Post deterioration has not brought back a repetition of the vegetational types which characterized the period immediately preceding the post-glacial climatic optimum.

Secondly, the actual resultant curve of climate is differently affected, in the manner illustrated by the difference between segments *EF* and *FG* in Fig. 6B. This point might offer some clue as to why the Granlund series, and perhaps also the smaller oscillations accompanying it, are seen in the later phases of the post-glacial record (represented, say, by section *GH* of the curve), but not seen, so far at any rate, in the earlier 'Atlantic' stage (represented, say, by section *FG*). It would also illuminate the suggestion made in discussing the complexities in the data of the late-glacial period; for if this period, say, from 12,000 B.C. to 6000 B.C., did really include a medium-scale oscillation such as that represented by section *EF*, then we might indeed expect to find within that period the expression of a multiple series of minor retrogressions.

4. SUGGESTIONS FOR EXTENDED INVESTIGATION

Let us formulate some of the questions which our investigations must be designed to answer:

(i) Supposing that a rhythm with a period of x years has been demonstrated in region A between dates T_1 and T_2, then, (*a*) can it be recognized elsewhere than at A, and (*b*) can it be recognized in periods not included between T_1 and T_2?

(ii) What suggestion, if any, can be made concerning the master factor in a given rhythm?

(iii) Does the master factor appear to be the same for all the rhythms which are found?

Turning to the practical methods for finding answers to these questions, we must stress the importance of variety, both in type of technique and in the place of investigation.

There is first of all the line opened up by von Post, namely, the detailed analysis variation in pollen curves. It appears that *Betula* has been the most suitable indicator the most recent peat layers, but total pollen content is also a valuable index. There must, however, be other species which would be suitable in other phases of the pollen diagrams. Thus, for example, something might come from close attention to the oak-alder pollen ratios of zone VII*b* of Pennine peats. Again, although *Sphagnum* spores in ombrogenous peats give a first impression of fluctuating rather wildly, there is just a possibility that some significance may be seen in them. Here, as in all these studies, the method depends on taking samples as closely spaced as the nature of the deposit will allow.

In making these analyses we have also to remember the kind of phenomenon that we may be handling. Welten (1944) has given considerable discussion of the point that the first effect of a climatic change will be on the pollen productivity of a given tree or stand of trees, and thus there may be a change in the pollen curve that does not reflect any alteration in the number of trees of that type. Such an alteration may follow in due course, but in the case of the smallest rhythms, it is more than likely that the only effect will be that on the pollen productivity.

Secondly, it seems desirable to push still further with the kind of micro-stratigraphy that is exemplified by the work of Granlund, Nilsson, and Ording. The banding of the upper layers in the north British blanket peats is conspicuous and, while it may have its explanation in the 'regeneration complex' structure, it will certainly be worth while examining it closely from the present point of view.

Then again, wherever it is possible, it is highly desirable to combine other types of estimation with the botanical investigation of post-glacial profiles. Varved deposits are unfortunately rare in this country, but Pennington's work in Windermere (Pennington, 1943, 1947; Pearsall & Pennington, 1947) deals with one such deposit, and also combines observations on diatoms and on other variables such as *C*/*N* ratio and so on. Welten again has produced results of the greatest interest from his analysis of pollen samples for organic matter and chalk content. Clearly, lake deposits offer a more varied field than ombrogenous peats, but even here something might come from the statistical treatment of such fossils as fungal spores and animal eggs.

Lastly, in considering the type of locality to choose for investigation, the question of sensitivity is all-important. Thus the reactions of a vegetation type to minor climatic fluctuations will be most marked in places near to the margin of the climatic region which is characterized by that vegetation type. We may well get some fresh evidence from the micro-stratigraphy of bogs somewhere in continental Europe just at the limits for ombrogenous peat formation. Again, within any one region, altitude variations may either lessen or enhance the expression of some particular climatic oscillation.

So one might continue giving examples, but the most profitable lines of attack have, of course, to be worked out by the individual investigator who has a particular area to study, or a particular 'rhythm' which has caught his interest. It is best that one should approach these problems without any influence from the present ideas of climatologists as to the climatic rhythms they may have detected within their own fields of observation. If the historical record, read with an open mind, does appear to give evidence of any rhythmic character in the behaviour of climates, then the data may be handed over to the climatologist, the meteorologist, and the astronomer, so that they may proceed to the further analysis of the physical phenomena. The hypothesis here discussed may seem to have a highly Copernican flavour, but if it leads to the collection of data it may help to prepare the way for some future Galileo of climatology.

REFERENCES

Clapham, A. R. & Godwin, H. (1948). Studies of the post-glacial history of British vegetation. VIII. Swamping surfaces in peats of the Somerset levels. *Philos. Trans.* B, **233**, 233.

Conway, V. M. (1947). Ringinglow bog, near Sheffield. *J. Ecol.* **34**, 149.

Cooper, W. S. (1942). Vegetation of the Prince William Sound region, Alaska; with a brief excursion into post-Pleistocene climatic history. *Ecol. Monogr.* **12**, 1.

Flint, R. F. (1947). *Glacial Geology and the Pleistocene Epoch.* New York.

Godwin, H. (1945). Coastal peat beds of the North Sea region, as indices of land and sea-level changes. *New Phytol.* **44**, 29.

Godwin, H. (1946). The relation of bog stratigraphy to climatic change and archaeology. *Proc. Prehist. Soc.* for 1946, Paper 1.

Godwin, H. (1947). The late-glacial period. *Sci. Progr. Twent. Cent.* **138**, 185.

Godwin, H. & Conway, V. M. (1939). The ecology of a raised bog near Tregaron, Cardiganshire. *J. Ecol.* **27**, 313.

Godwin, H. & Mitchell, G. F. (1938). Stratigraphy and development of two raised bogs near Tregaron, Cardiganshire. *New Phytol.* **37**, 425.

Granlund, E. (1932). De Svenska Högmossarnas Geologi. *Sverig. geol. Unders. Afh.* **26**, Ser. C, no. 273.

Hardy, E. M. (1939). Studies of the post-glacial history of British vegetation. V. The Shropshire and Flint Maelor mosses. *New Phytol.* **38**, 364.

Härri, H. (1940). Stratigraphie und Waldgeschichte des Wauwilermooses. *Veröff. geobot. Inst. Rübel.* **17.**

Iversen, J. (1947). Plantevaekst, Dyreliv og Klima i det senglaciale Danmark. *Geol. Fören. Stockh. Förh.* **69**, 67.

Lüdi, W. (1944). Die Waldgeschichte des südlichen Tessin seit dem Rückzug der Gletscher. *Ber. Geob. Forsch. Inst. Rübel.* (1943), 12.

Nilsson, T. (1935). Die pollenanalytische Zonengliederung der spät- und postglacialen Bildungen Schonens. *Medd. Lunds geol.-min. Instn.* **61.**

Pearsall, W. H. & Pennington, W. (1947). Ecological history of the English Lake District. *J. Ecol.* **34**, 137.

Pennington, W. (1943). Lake sediments; the bottom deposits of the North Basin of Windermere, with especial reference to the diatom succession. *New Phytol.* **42**, 1.

Pennington, W. (1947). Studies in the post-glacial history of British vegetation. VII. Lake sediments; pollen diagrams from the bottom deposits of the north basin of Windermere. *Philos. Trans.* B, **233**, no. 596, p. 137.

von Post, L. (1944). Pollenstatistika perspektiv på Jordens klimathistoria. *Ymer* 2. *Medd. Stockh. Högsk. geol. Inst.* no. 64.

von Post, L. (1946). The prospect for pollen analysis in the study of the earth's climatic history. *New Phytol.* **45**, 193.

Welten, M. (1944). Pollenanalytische, stratigraphische, und geochronologische Untersuchungen aus dem Faulenseemoos bei Spiez. *Veröff. geobot. Inst. Rübel.* **21.**

Wright, W. B. (1937). *The Quaternary Ice-age.* London.

STUDIES ON PLANKTON PARASITES

I. FLUCTUATIONS IN THE NUMBERS OF *ASTERIONELLA FORMOSA* HASS. IN RELATION TO FUNGAL EPIDEMICS

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(With 11 figures in the text)

CONTENTS

	PAGE
A. Introduction	238
B. Methods	239
C. Parasitism	240
D. The lakes and the periodicity of *Asterionella*	241
E. *Rhizophidium planktonicum* and the periodicity of *Asterionella*	242
(i) Late summer to early spring	242
(ii) Early spring to summer	250
F. Developmental phases of *Rhizophidium* in relation to epidemics	252
G. Behaviour of *Rhizophidium* under artificial conditions	252
H. External factors and the onset and course of epidemics of *Rhizophidium planktonicum* in nature	255
I. Taxonomy of *Rhizophidium planktonicum* n.sp. (by H. M. Canter)	257
J. Summary	260
References	260

A. INTRODUCTION

No critical investigation has been made on the effect of parasitic fungi in relation to fluctuations in the numbers of planktonic algae. Wesenberg-Lund (1905, p. 436—with reference to plankton algae) says his observations 'prove that an organism in the latter part of the period of maximum development may very often be infected by Phycomycetes which feed upon the protoplasm and kill it, leaving the skeleton intact'. Reynolds (1940) showed that a chytridiaceous fungus reduced the numbers of a form of *Staurastrum paradoxum* Meyen. Though several other workers (e.g. Huber-Pestalozzi, 1946; de Wildeman, 1900, 1931) describe chytridiaceous fungi parasitizing plankton algae, they give no indication whether or not they exert any marked effect on the algal population. Weston (1941, esp. p. 142) has reviewed the role of aquatic fungi in hydrobiology and stresses the probable importance of parasites in controlling the numbers of plankton algae.

The majority of the larger planktonic algae of Windermere, Esthwaite Water and Blelham Tarn, lakes in the southern part of the English Lake District, are parasitized by fungi (mainly Chytridiales). Cursory observations in the autumn of 1945 suggested that, at certain times, the fungi appreciably reduced the numbers of, among others, *Asterionella formosa* Hass. During an epidemic* the number of live *Asterionella* cells fell from 415 to 57 per ml. in Windermere (south basin). Since it was obvious that other factors might be reducing the algal numbers at the same time, a detailed study of the population of this diatom was made in 1946, 1947 and 1948 the results of which are here described.

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B. METHODS

Collections were made weekly in the north end of Esthwaite Water, the east end of Blelham Tarn, and the south and north basins of Windermere. The depths at these collecting stations are 15, 12, 34 and 60 m. respectively. During periods when fungal epidemics were expected or did occur, collections, so far as possible, were made every 2–4 days. A column of water from 0–5 m. was collected with a rubber tube. A 5 m. length of rubber tube was weighted at one end and a cord somewhat longer than the tube was attached close to the weight. After thorough rinsing with the lake water, the tube was lowered slowly into the water until it was hanging vertically with the unweighted end level with the water surface, the free end of the cord being retained at the surface. The lowering did not take less than 1 min., with a tube of 2·5 cm. internal diameter. The upper end was closed and the lower drawn up by the cord. When both ends were above water, the rest of the hose was lifted out as the contents were poured into a sample bottle previously washed with the lake water.† The algal numbers were obtained by sedimentation with a saturated solution of iodine in potassium iodide and counting on an inverted microscope by the Utermöhl (1931) technique. Counts of living *Asterionella* cells were obtained by counting 75 or more (usually over 100) colonies, except occasionally, when there were less than about 5 cells per ml. Sedimentation of samples over 100 ml. commonly leads to inaccuracies due to the large amount of other algae and sediment present. There are various sources of error involved in sampling, sedimenting and counting which will be considered elsewhere. Often they tend to cancel each other out, but it appears that counts of about 75 colonies will not vary more than approximately 20% from the mean found by taking twenty samples or twenty counts on one sample.

It is impossible, by visual inspection, to tell if a cell with contents is dead. Death was considered to have occurred when either the cell was empty or the contents (e.g. remains of chromatophores) had completely lost their morphological characteristics (Fig. 10B where the chromatophore remnants appear as round granules).

The *Rhizophidium* cells were counted on 100, or, during epidemics, 200 diatom colonies. Counting was done on fresh material, directly after its collection by net haul at the same time and place as the tube samples. Very rarely these counts had to be made on material preserved in glacial acetic acid and absolute alcohol. As well as determining the number of parasitized and living cells in each *Asterionella* colony, the total number of zoospores, living sporangia, empty sporangia and resting spores of *Rhizophidium* present were analysed separately. The term zoospore refers to the recently encysted swarmers attached to the host wall which, though they may have grown slightly, still contain the original single oil globule. Larger cells, at a later stage of development, which contain many oil globules or incipient zoospores are classed as living sporangia.

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* The fungi may parasitize the algae concerned to a small extent over prolonged periods and the term epidemic refers to the occasional outbursts of severe parasitism.

† This method, which ensures a uniform sample from the 0–5 m. water column, was suggested by Mr H. J. Buchanan-Wollaston.

C. PARASITISM

The prolonged observation of large numbers of infected cells shows clearly that *R. planktonicum* is a parasite and not a saprophyte. Healthy cells of *Asterionella* possess well-defined parietal chromatophores covering a considerable proportion of the surface of the cell (Fig. 10A). Single zoospores are frequently present on such cells (Fig. 10A, *x*), but when well-developed or empty sporangia or resting spores are present, the chromatophores are either considerably disorganized (Figs. 10B, 11G–J, O) or wholly destroyed. At this stage, too, the internal (endobiotic) rhizoidal system of the fungus is nearly always *clearly* visible. Similar changes in the appearance of the cell content occur when the cells die from other causes and it is noteworthy that, when *Rhizophidium* is present, it is the healthy rather than visibly dying cells which most frequently bear the zoospores. Indirect evidence of parasitism is shown in two other features. First, when *Rhizophidium* is present, it does not always show any marked increase in numbers when the diatom population dies from other causes (cf. Fig. 6, May–June; Fig. 8) as might be expected if this was a saprophytic fungus. This is particularly evident at the end of the spring maximum of *Asterionella*. At such times, the number of live *Asterionella* cells falls from between 5000 and 12,000 to 10 or less per ml. in a few weeks. Secondly, the number of live cells per colony in *Asterionella* is well known to vary (cf. Gardiner, 1940–1; Pearsall, Gardiner & Greenshields, 1946), particularly during periods of active growth. It falls as environmental conditions become less and less favourable. Irregular small fluctuations occur under all conditions. The number of cells per colony may also be expected to fall during a period of severe fungal infection and this has always been observed (Figs. 1–6). The ratio of dead to living cells also rises in such epidemics. Flood periods often coincide to some extent with periods of high fungal parasitism and can reduce the numbers of diatoms (p. 241). Floods should not, however, reduce the average number of cells per colony appreciably since there is no apparent reason why they should impair the vigour of the colonies. Indeed, the reverse might be expected since the inflows carry nutrients in solution. Mechanical disintegration of the colonies by water turbulence is not necessarily any more likely during flood periods than at other times, since turbulence of lake water is predominantly a wind effect. Moreover, even shaking cultures in flasks, when the colonies will often strike the walls, needs to be prolonged and severe to disintegrate healthy colonies. The average number of cells per colony (hereafter referred to as 'cells per colony') is generally high in autumn and winter, the main flood period. Fig. 4, 12 November–14 January, which illustrates a fall in *Asterionella* numbers correlated with high lake levels, supports this view, since, after the original fall from a very high cell per colony average of 8·2, the average number of cells does not vary significantly, being always above 6 as can be seen by comparing Fig. 4 with Fig. 1, 2–15 November; Fig. 2, 23 October–1 November. In all cases (Figs. 1–6), irrespective of lake level, the end of a severe fungal infestation is correlated with a rise in the average number of cells per colony. This feature can, therefore, be used as indirect evidence of the effect of severe parasitism and of its occurrence in periods synchronous, to a greater or less degree, with high lake levels and consequent reduction in the numbers of *Asterionella* from loss by outflow.

Huber-Pestalozzi (1946) states that the cell walls of *Asterionella* may be deformed as a result of parasitism by chytridiaceous fungi. We have never observed this. Very occasional deformed walls do occur but without any relation to the presence or absence of *Rhizophidium*.

D. THE LAKES AND THE PERIODICITY OF *ASTERIONELLA*

The north and south basins of Windermere are separated by an area of relatively shallow water containing a group of islands (Mill, 1895). Though qualitatively similar in chemistry and biology, they differ quantitatively. They have, therefore, been treated separately. In general, the south basin which, besides north-basin water, receives the outflow of Esthwaite Water (*q.v.*) and sewage from the main centres of population (Windermere town and Bowness) adjacent to the lake, is more productive and eutrophic (for definition of this and other limnological terms see Welch, 1935) in nature than the north basin. Maps of Esthwaite Water and Blelham Tarn are given by Mortimer (1941, p. 284; 1942, p. 180). They are both somewhat silted and eutrophic, but while the phytoplankton of Esthwaite Water is dominated by Myxophyceae and diatoms, that of Blelham Tarn is dominated by Chrysophyceae and diatoms. Windermere south basin is similar to Esthwaite Water, apart from greater numbers of desmids and colonial Chlorophyceae. Windermere north basin is the most oligotrophic of the four bodies of water and contains fewer Myxophyceae than the south basin and, especially, Esthwaite Water and fewer flagellates than Blelham Tarn.

Asterionella formosa Hass.* is the dominant diatom in the plankton of the three lakes over a considerable part of the year. There is a large spring and a small autumn maximum, reaching 12,000 and 400 cells per ml. respectively. In Windermere north basin the autumn maximum is very small (16 or fewer cells per ml.). The onset of the spring increase appears to be determined by the increasing amount of daylight from February to April, and its end is correlated with a drop in silica concentration from about 2–3 to 0·5 or less mg. per litre in May or June. The autumn increase starts in late summer or early autumn when thermal stratification begins to break down and the silica and nitrate-nitrogen concentrations rise. Several factors,† besides fungal parasitism, may limit the size of the autumnal increase and the numbers during winter, notably:

(1) Light duration and intensity, both of which are falling.

(2) *Turbulence*. The indirect effect of falling temperature which by lowering and finally destroying the thermocline increases the depth to which turbulence is effective. Thus there is a greater chance of cells being carried out of the photic zone for long periods or permanently. This effect is particularly important in deep lakes such as Windermere north basin (maximum depth 66 m.).

(3) *Floods*. In smaller lakes, such as Blelham Tarn and Esthwaite Water, floods wash out relatively larger numbers than in Windermere, where only the most severe have any marked effect. Flood effects are twofold. The lake level is heightened and so the number of cells at a given depth is decreased by dilution. The total number in the lake, however, would remain the same, were it not for the increased loss by outflow. This second effect is the more important. The relative degree of loss by outflow depends on the ratio of inflow to lake volume. The severity of the flood effect in any lake depends largely on the

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* There is much confusion between this species and *A. gracillima* Heib. It is not clear whether there are two separate species. The vast majority of the present specimens agree with Hustedt's (1938, pp. 251–2) definition of *A. formosa*. There is no doubt that many records of *A. gracillima*, including all those for the English Lake District, refer mainly to *A. formosa* sensu Hustedt.

† The data on which some of the views expressed below are based will be described elsewhere.

size and speed of increase in lake level (and so of outflow), though naturally flow is greater at high than at low lake levels. The temperature of the inflowing water relative to that of the lake is important since it controls the depth to which it will pass on entry into the lake. Under stratified conditions inflowing water may, for example, pass into the zone where *Asterionella* is multiplying or below it. In the former case the inflowing water will apparently reduce the numbers of *Asterionella* by dilution and really reduce them through increased loss by outflow; it will, however, also add new supplies of nutrients. In the latter case, the inflowing water will have no effect at all on the numbers of *Asterionella* in the upper layers (e.g. 0–5 m. depth).

Flood effects are particularly severe in winter when the reduced light (and possibly temperature) leads to a slow rate of growth. In Blelham Tarn (and Esthwaite Water) the rise and fall in level parallels that in Windermere. Fig. 4 (12 November–14 January) illustrates the reduction in numbers of *Asterionella* in Blelham Tarn during November to January, 1946–7, when *Rhizophidium* numbers were low and concentrations of dissolved salts high. This reduction can then be correlated with high lake levels. A very similar reduction in *Asterionella* and rise in lake level took place in November 1947. From 14 to 24 November 1946 there was a rise in level of 1·07 m. (3½ ft.) and a drop in cell numbers from 180 to 20 per ml. Two further marked rises in level occurred (21–27 December, 10–17 January) and, throughout the period, the level was near or above 130 ft. which is well above the yearly mean (129·2 ft.). Such high levels are only attained after periods of heavy rainfall. By 14 January there were only about 2 cells per ml.

(4) *Ingestion.* The importance of this factor has not been studied in detail, but only one of the larger zooplanktonts (*Asplanchna priodonta* Gosse)* has been observed ingesting *Asterionella* colonies. Examination from time to time of the common species of *Bosmina*, *Diaptomus*, *Daphnia*, *Cyclops* and the smaller Rotifera has failed to produce any evidence that they ingest so large a colony. Single cells are sometimes present in gut contents and fragments of cells quite commonly. Single live cells are, however, so rare that even if all of them were ingested the *Asterionella* population would never be appreciably reduced. *Asplanchna* was obtained in the largest numbers in Blelham Tarn but occurred in all three lakes in the summer and early winter of 1946 and 1947. It ingested all the plankton algae apparently without selection. During this period the numbers of *Asterionella*, *Melosira*, *Fragilaria* and *Tabellaria* all rose considerably. Though there are no data as to the numbers of *Asplanchna*, it does not appear that it stops the population increasing but, at the most, reduces the rate of increase.

(5) *Competition* with Myxophyceae in Windermere south basin and Esthwaite Water and with other diatoms in all three lakes. There is no evidence that the Myxophyceae inhibit the growth of the diatoms or that the latter inhibit each other's growth. They may compete for nutrients but, in the periods when the main epidemics occurred, the concentrations of silica, nitrates and phosphates were all rising, or at or near their seasonal maximum.

E. *RHIZOPHIDIUM PLANKTONICUM* AND THE PERIODICITY OF *ASTERIONELLA*

(i) *Late summer to early spring*

This period, in which most of the *Rhizophidium* epidemics occurred is, for that reason, considered first. It covers the period between the renewal of growth of *Asterionella* after the summer minimum, to the beginning of the spring-growth period. Detailed data are available for 1946, 1947 and 1948 and general observations for 1945.

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* We are indebted to Mr D. J. Scourfield for naming this rotifer.

Esthwaite Water (Figs. 1–3). The autumn growth* period of *Asterionella* began between 24 September and 1 October 1946 (Fig. 1). The numbers of *Rhizophidium* remained low until 28 October when 10 % of *Asterionella* cells were infected. There were then 322 live *Asterionella* cells per ml. Five days later the infection had risen to 26 % and *Asterionella* to 390 cells per ml. Two days later (4 November) infection was 2 % lower and the numbers of *Asterionella* were virtually unchanged (388 per ml.). On 8 November

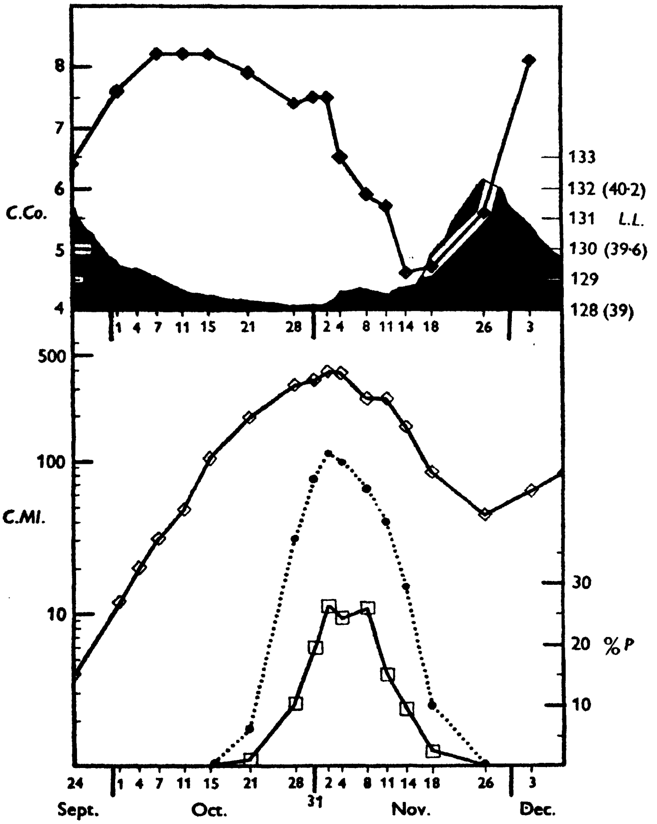

Fig. 1. Esthwaite Water, autumn 1946. Relationship between the numbers of *Asterionella formosa* and its parasite *Rhizophidium planktonicum*. In this and Figs. 2–8 inclusive, except where noted to the contrary, the following abbreviations and representations are used: *C.Co.*, average number of live cells per *Asterionella* colony (a solid line with black diamonds); *C.Ml.*, number of live *Asterionella* cells per ml. plotted on a log scale (a solid line with white diamonds) and the number of live *Rhizophidium* cells per ml. plotted on a log scale (a dotted line with black circles); *L.L.*, Windermere north basin lake level in feet (metres in brackets) shown solid black; *%P.*, percentage of *Asterionella* cells infected by living *Rhizophidium* cells (a solid line with white squares). The diamonds, squares and circles mark the dates on which estimations were made. The dates themselves lie along the base of the figure and also below the base line of the lake level; the last day of each month is marked by a longer and thicker line than the other dates.

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* The term 'growth' is here used for the increase in numbers of a population and not for any increase in cellular dimensions.

parasitism was again 26% of the *Asterionella* cells whose number had declined to 266 per ml. From then to 26 November, the numbers of *Asterionella* (to 45 cells per ml.), and *Rhizophidium* (to less than 1% infection) decreased. From 18 to 26 November there was a rapid rise in lake level,* and a part of the final fall in *Asterionella* numbers may be attributed to mechanical depletion. In addition, though there are no temperature data for Esthwaite Water, there is strong indirect evidence that this was also the period of final and complete loss of temperature stratification, so that some dilution may have occurred also due to vertical mixing.

The variation in the number of *Asterionella* cells per colony showed a marked correlation with the course of the *Rhizophidium* epidemic. With the start of the autumn-growth period, the cell per colony average rose from 6·4 to over 8 and then declined slightly to the still high level of 7·5 but, by 14 November it had fallen to the very low level of 4·6. With the end of the *Rhizophidium* epidemic and the onset of flood conditions, the cell per colony average rose rapidly. Thus, as in the *Rhizophidium* epidemics which occurred in 1945, 1947 and 1948, the rise in numbers of the fungus was followed by a fall in the numbers of *Asterionella* and of cell per colony average while, until the end of the period, the lake level remained low. Throughout the period the concentrations of nitrates, phosphates and silica were within the limits suitable for active growth of *Asterionella*.

In 1947 (Fig. 2) a similar epidemic occurred in the same period and under closely similar conditions. The epidemic was of shorter duration, but the maximum infection (38% of the *Asterionella* cells) was higher. The number of *Asterionella* cells fell from 121 on 25 October to 37 on 6 November, while the cell per colony average fell from over 8 to under 4. Directly after the epidemic, the number of cells per colony began rising and remained over 7 throughout the flood periods which followed (8–13 and 20–24 November). These floods kept the *Asterionella* numbers at a low level, though a small rise took place between them (20 November). With the return of dry weather and continued paucity of *Rhizophidium*, the *Asterionella* numbers rose steadily (to 121 cells per ml. on 11 December). The number of dead *Asterionella* cells in this epidemic rose from 1 on 16 October to 61 on 29 October, and then decreased to 3 per ml. on 27 November. The ratio of live to dead cells fell from 12:1 on 16 October to 1·2:1 on 31 October and then rose to 17·1:1 on 27 November.

These two epidemics illustrate most clearly the effects of parasitism. The further ones to be described shortly have their effects, apart from the reduction in the number of cells per *Asterionella* colony, more or less masked by other effects (e.g. floods). It should, further, be realized that, in these two epidemics described above, the numbers of *Asterionella* would have increased in the absence of the severe parasitism. Judging by the rate of increase of the population before the epidemic began, this increase would have been substantial. It seems not unreasonable to believe that the numbers would have been about 500–1000 per ml. by the time the floods started, instead of 37 (Fig. 2, 6 November) and 85 (Fig. 1, 18 November).

In 1946, from the end of the epidemic first described (Fig. 1) until 30 December, there were few *Rhizophidium* cells. The numbers of *Asterionella*, after the rise consequent on the reduction of parasitism (Fig. 1, p. 243), remained more or less static, which is not surprising in view of the low light intensity and duration and high lake level. The number of cells per colony, after the first sharp rise to 8·1, then declined somewhat, but not below 7 (Fig. 3). By 23 December a new *Rhizophidium* epidemic had started, 32% of the *Asterionella* cells being infected on 5 January 1947. By 13 January, despite no marked change in lake level, the number of *Asterionella* had fallen from 113 (3 January) to 56 per ml., 27% of the cells being infected. The final fall in *Asterionella* to 27 cells per ml. on 20 January was correlated with a rapid rise in lake level, but the average number of cells per colony points to parasitism also being a partial cause of the decrease. From the original 7 (23 December), the number of cells per colony dropped to 4·3 on 20 January.

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* As in Blelham Tarn, comparative observations show that the lake level fluctuates similarly to that of the north basin of Windermere, for which daily records are available.

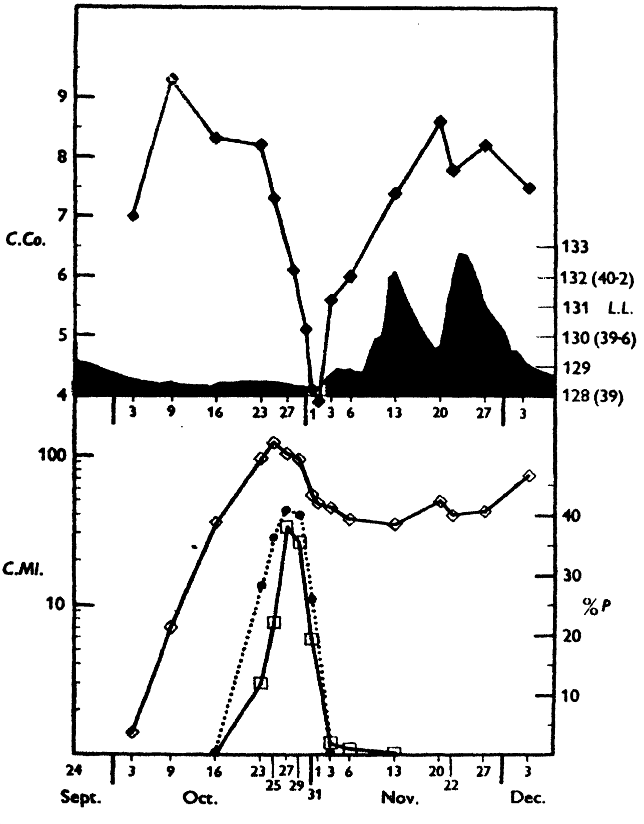

Fig. 2. Esthwaite Water, autumn 1947. For explanation see Fig. 1.

With the fall in infection, together with dry weather and increasing illumination, the numbers of *Asterionella* reached 229 per ml. on 11 February. Shortly afterwards the relatively rapid increase characteristic of the spring-growth period began. The numbers of cells per colony also rose from 4·3 (20 January) to 6·9–7·0 (4–11 February). Whereas this rise after the two epidemics previously described (Figs. 1 and 2) occurred during a period of rising lake level, in this case lake level was falling. This supports the view (p. 240) that the average number of cells per colony is not affected by changes in lake level.

A similar though less severe (maximum infection 19% of the *Asterionella* cells) epidemic occurred between 22 December 1947 and 15 January 1948.

Blelham Tarn. A severe but very short *Rhizophidium* epidemic began between 3 and 10 September 1946 (Fig. 4), 41 % of the *Asterionella* cells being infected by 14 September. The numbers of *Asterionella* had fallen during a sharp rise in lake level (29 August–6 September) before the epidemic. Though the level remained relatively high, it was falling slowly between 12 and 21 September when the *Asterionella* cells fell from 47 to 6 per ml. The changes in the average number of cells per colony support the view that this fall was, in part at least, due to fungal infection. Between 22 August and 10 September there were 5·6–6·3, and by 14 September only 4·2 cells per colony. The final reduction in cell numbers to 4 per ml. (23 September) and a new fall in cells per colony from 5·0 to 4·4 (17–23 September) were due to a new flood period (21–23 September) and the breakdown of thermal stratification. The latter brought into the upper water (e.g. 0–5 m.) colonies with few cells from the thermocline and hypolimnion. With the end of the epidemic, dry weather and isothermal conditions, the numbers of cells per ml. and of cells per colony of *Asterionella* rose steadily (23 September–5 November). The later fall in numbers during a period of high lake level and low illumination has been discussed earlier (p. 242).

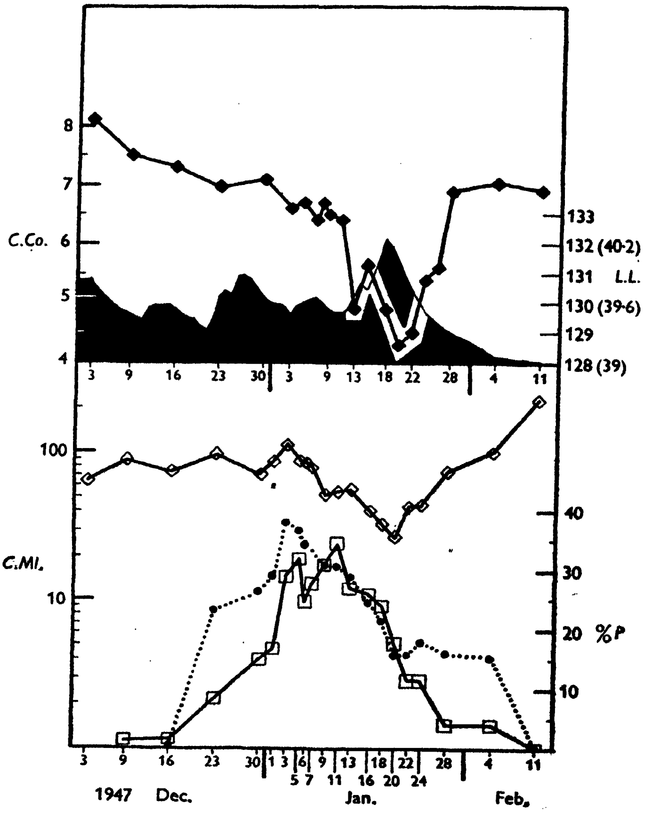

Fig. 3. Esthwaite Water, winter 1946–7. For explanation see Fig. 1.

In 1946 (January, Fig. 5) and 1947 (March, Fig. 6) *Rhizophidium* epidemics occurred in Blelham Tarn, the effects of which were more or less masked by ice and snow and floods.

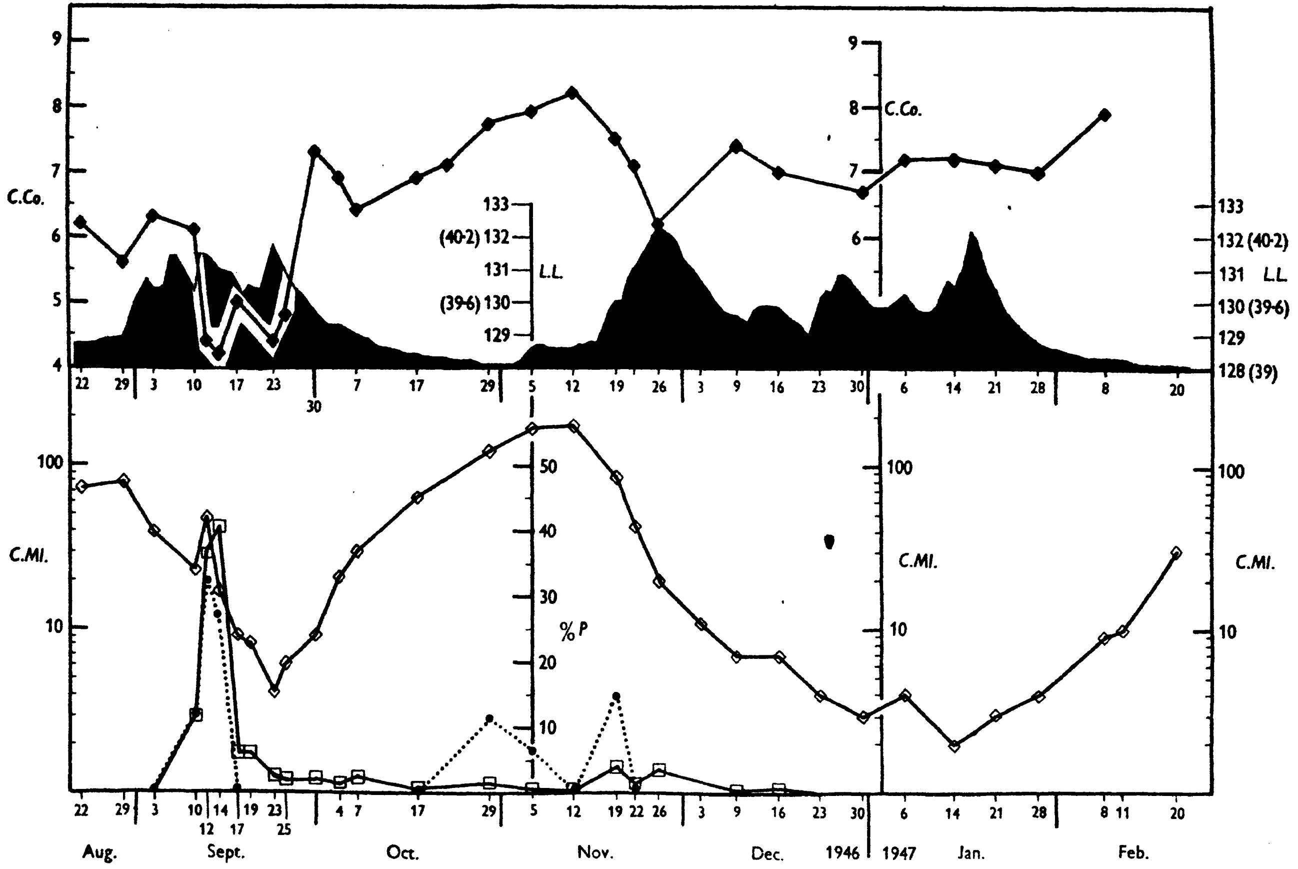

Fig. 4. Blelham Tarn, autumn–winter 1946–7. For explanation see Fig. 1. The numbers of *Rhizophidium* per 10 ml. are shown instead of per 1 ml. as in Figs. 1–3, 5–8. From November 1946 onwards the graph illustrates the relationship between the numbers of *Asterionella* and the fluctuations in lake level (see p. 242).

Ice-cover alone tends to increase rather than decrease the growth rate of *Asterionella* in the photic zone. Light penetration is practically the same as in open water. The diminution of water movement, due to the cutting off of wind action, keeps the cells, originally present, in the photic zone. Snow lying on the ice, however, cuts down light penetration and retards growth.*

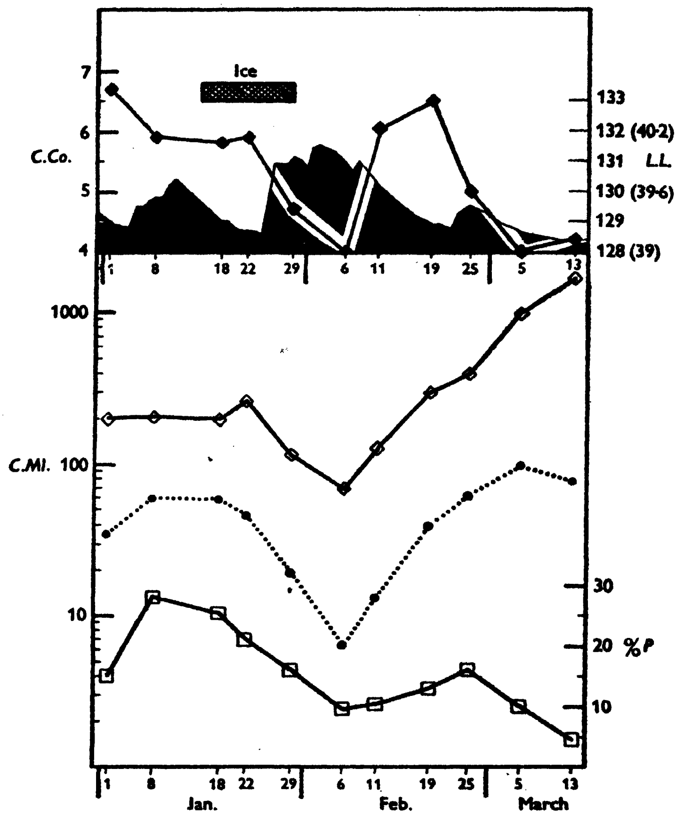

Fig. 5. Blelham Tarn, winter–spring 1946. For explanation see Fig. 1. The period of ice-cover is indicated at the top of the graph.

The 1946 epidemic was the first examined in detail and later work makes it clear that the intervals between the collections were too long. The epidemic period was, however, relatively long. Snow was present in such small amount and for so short a time on the ice that it can hardly have caused the virtual absence of growth between 1 and 22 January and the fall in *Asterionella* numbers from 210 to 65 per ml. between 22 January and 6 February. The latter appears to be mainly due to the flood (24 January–2 February). That it is not entirely due to this is suggested by the fall in the average number of *Asterionella* cells per colony from 6·7 (1 January) and 6·4 (22 January) to 4 (6 February). When fungal infection fell to 10% and dry weather started (11 February) the numbers of cells and cells per colony of *Asterionella* rose sharply. The further fall in the number of cells per colony (19 February–13 March) is not only due to the rise in parasitism to 16% (25 February), but also to the usual course of the spring maximum (p. 240).

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* Data supporting this interpretation will be published at a later date.

The 1947 epidemic (Fig. 6) was synchronous with high lake level combined with prolonged thick snow lying on ice. The whole ice-sheet rose with the lake level. That the final drop in *Asterionella* numbers was due in part to parasitism is suggested by the much larger decrease than in Esthwaite Water, where the same ice, snow and floods occurred, and by the sharp drop in the number of cells per colony (5·7 on 19 March; 4·2 on 29 March). Further, after the epidemic, the numbers of cells per ml. and per colony rose sharply, as in the other epidemics.

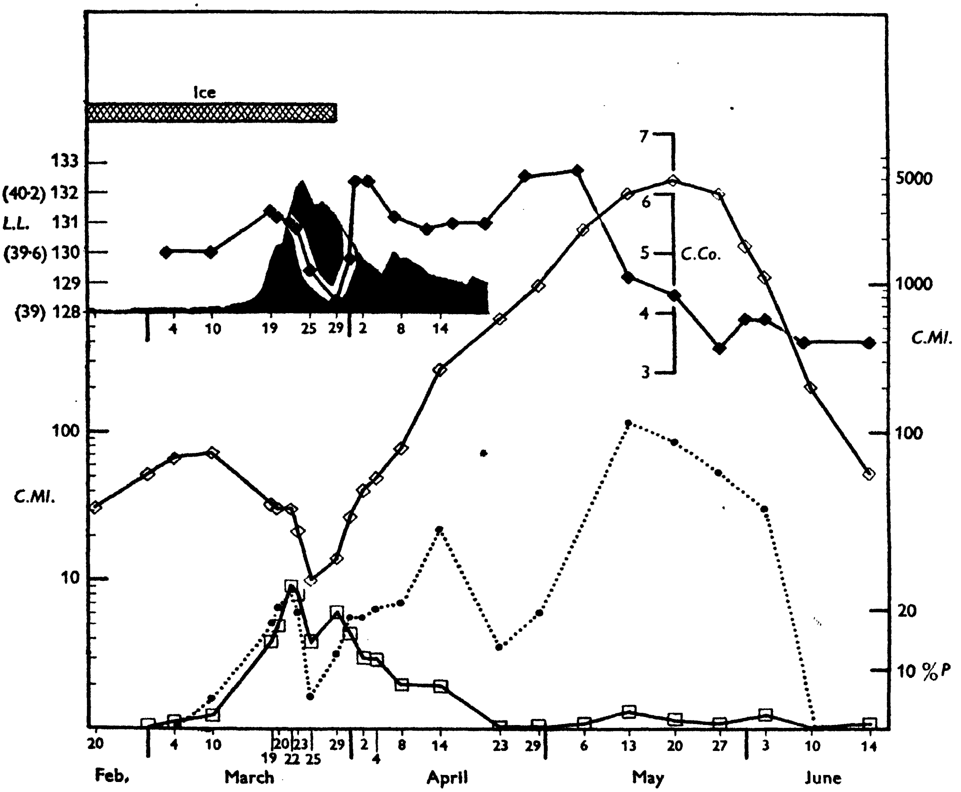

Fig. 6. Blelham Tarn, spring 1947. For explanation see Fig. 1. The lake level is discontinued after 21 April since the remainder of the graph illustrates the course of a spring-growth period of *Asterionella* free of fungal epidemics (see p. 241). The period of ice-cover is indicated at the top of the graph.

Rhizophidium occurred in moderate numbers during November 1945, but no counts were made. In 1947 numbers were low throughout autumn and early winter.

Windermere south basin. In 1946 and 1947 no such *Rhizophidium* epidemic occurred as in 1945 (p. 239). Three small rises in the fungal infection occurred (12, 6 and 16%) but, though each was followed by a fall in the number of *Asterionella* cells per colony, the number of cells per ml. was not significantly altered.

Windermere north basin. Neither the numbers of *Asterionella* nor of *Rhizophidium* increased to any large amount during this period in 1945, 1946 or 1947.

(ii) *Early spring to summer*

Only two epidemics occurred in this period. The one in Blelham Tarn has been described above (p. 249, Fig. 6) but the light and temperature conditions were those of winter. The other occurred after the spring maximum of *Asterionella* in Esthwaite Water (Fig. 7).

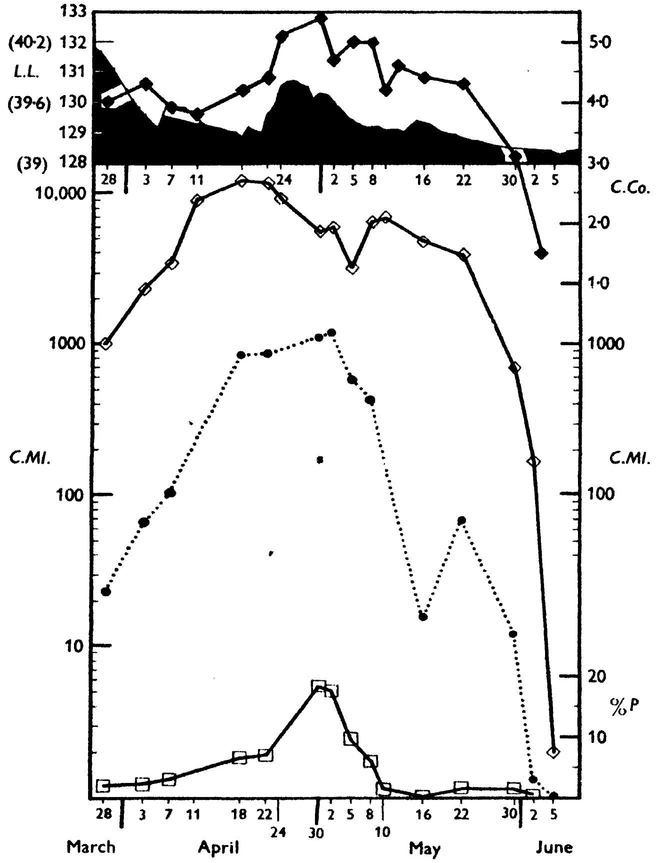

Fig. 7. Esthwaite Water, spring 1947. For explanation see Fig. 1.

The numbers of *Rhizophidium* remained almost constant from 18 April to 2 May, while the numbers of *Asterionella* fell sharply after the maximum infection had been reached (30 April, 18 %). The change in cells per colony was not marked but, as is usual, the number was already very low at the end of the *Asterionella* growth period. An additional complexity, in evaluating the effect of the various external factors on *Asterionella*, lies in the flood period (22–26 April). The inflow water was warm relative to that of the lake, and consequently passed into the surface layers reducing the numbers of *Asterionella* by mechanical depletion (p. 241). The number of cells per colony rose, however, in response to the added nutrients. It appears that, in the absence of the epidemic, the post-maximal decline in *Asterionella* numbers would have been relatively slow till 22 May, due to this replenishment. When *Asterionella* cells are dying in large numbers, but often before they are visibly dead (p. 239), a growth of characteristic bacteria occurs on the frustules, but this did not become prominent until 22 May. From 16 May hot dry weather reduced the inflow, the population of *Asterionella* decreased sharply, as did the number of live cells per colony.

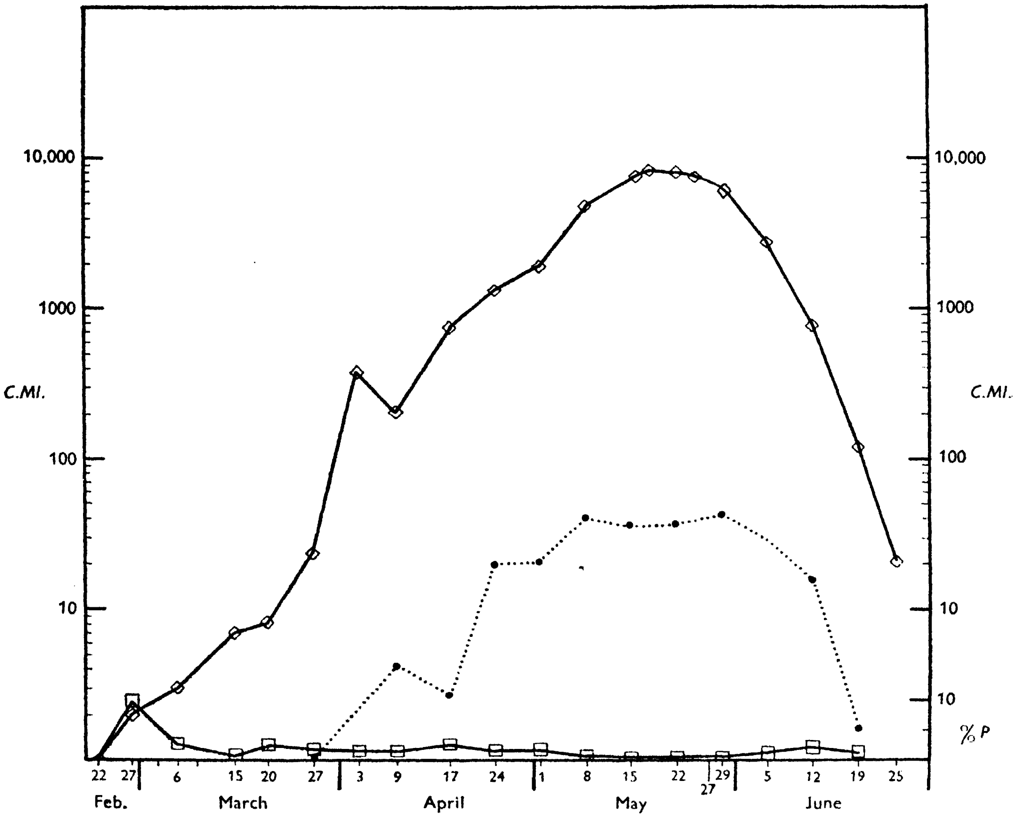

Fig. 8. Windermere north basin, spring 1946. For explanation see Fig. 1. Lake level (*L.L.*) and the average number of live cells per *Asterionella* colony (*C.Co.*) are omitted since this figure illustrates a period in which no *Rhizophidium* epidemics occurred (see p. 242).

It is remarkable that no epidemics occurred in any of the four bodies of water in the three years during the main part of the spring-growth period. The usual course of events is illustrated in Fig. 6, April–June, and Fig. 8, and discussed further on p. 241. In addition only once did an epidemic occur after the *Asterionella* maximum, and this at a time when the typical catastrophic fall in numbers of live cells had not started. This is strong indirect evidence against the fungus being a saprophyte (see also p. 240).

*Rhizophidium** has also been observed on *Asterionella* in Crummock Water, Buttermere and Bassenthwaite Lake, lakes in the English Lake District. In April 1947 a visit was made to Loweswater, a lake somewhat similar to Esthwaite Water. While at this time, the number of *Asterionella* cells per ml. in Esthwaite Water was 3900, in Loweswater it was only 17 per ml. The difference may be due, in part, to the fact that there was a severe epidemic in Loweswater, over 30% of the *Asterionella* cells being parasitized.

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* As resting spores have not been observed in material from other lakes the identity with *Rhizophidium planktonicum*, though probable, is not proved.

In Malham Tarn, Yorkshire, England, *Asterionella* is parasitized by another chytridiaceous fungus (? *Zygorhizidium* sp.).

F. DEVELOPMENTAL PHASES OF *RHIZOPHIDIUM* IN RELATION TO EPIDEMICS (Fig. 9)

The natural sequence of an epidemic can be followed from an analysis of the stage in development of each fungal cell (i.e. encysted zoospore, sporangium, empty sporangium and resting spore). The sequence in Esthwaite Water from October 1946 to February 1947 is given as an example (Fig. 9). In the early stages of an epidemic, attached encysted zoospores predominate and, though a high percentage of infection is therefore recorded, no reduction is seen in the algal numbers. This is because the chromatophores are as yet little disorganized and a cell, though destined to die, is not yet definitely dead. When the period of active fungal multiplication ends, for reasons unknown, there is a decrease in the number of zoospores while the number of sporangia still increases. In the last stage, very few reinfective bodies (zoospores or sporangia) remain while the empty sporangia reach a maximum. The algal numbers now fall sharply.

The factors governing the appearance of resting spores are obscure, but their appearance corresponds with periods of high fungal activity. On *Asterionella* in Esthwaite Water in 1946 they appeared at an early stage in the epidemic and reached a maximum towards its end. Similarly, they occurred in Blelham Tarn after epidemic peaks in spring and autumn, 1946, and in Windermere south basin, at the two highest points of fungal parasitism during 1946, though the actual percentage of infection observed was never more than 16%. Germination was not seen. Periods when no infection occurs are presumably passed in the resting spore stage, but it is noteworthy that only rarely are collections of *Asterionella* completely free of *Rhizophidium* zoospores or sporangia. Thus in 45 weeks in Blelham Tarn only three collections lacked *Rhizophidium.* In Esthwaite Water five out of 39 weeks; in Windermere north basin, four out of 41 weeks and in the south basin two out of 39 weeks produced samples free of *Rhizophidium*. It would appear therefore, that, at practically all times, *Rhizophidium* cells are present which might produce an epidemic. It is possible, however, that the epidemic-producing populations arise from the products of the germination of the sexually formed resting spores (cf. p. 256).

G. BEHAVIOUR OF *RHIZOPHIDIUM* UNDER ARTIFICIAL CONDITIONS

All attempts to cultivate *Rhizophidium* failed. *Asterionella* populations containing parasitized cells were placed in bottles or test-tubes in the laboratory and in the lake, using culture solutions (Chu, 1942, solution no. 10) or lake water. The samples were also subjected to varied conditions of light, aeration and temperature. Nevertheless, *Rhizophidium* failed to develop on the resultant *Asterionella* populations. Two quantitative experiments were performed (Table 1). In these, net samples of plankton were diluted in tap water and left in the laboratory at approximately 19° C., with poor illumination. At the same time, samples of lake water obtained by means of a rubber tube (see p. 239)

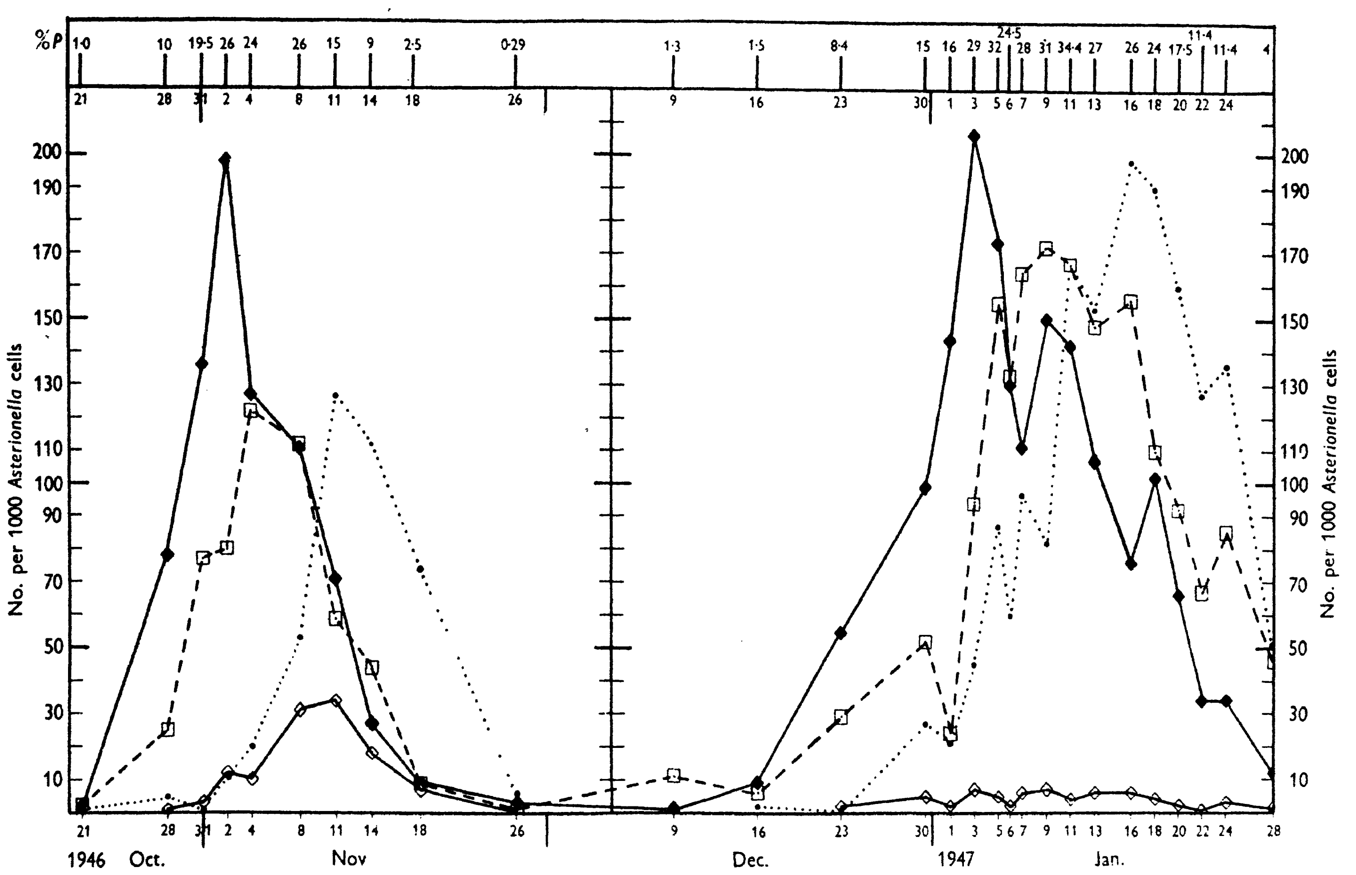

Fig. 9. Esthwaite Water, autumn-winter 1946–7. Developmental phases of *Rhizophidium* during two epidemics. Zoospores, solid line with black diamonds; sporangia, interrupted line with white squares; empty sporangia, dotted line with black circles; resting spores (zygotes), solid line with white diamonds. The number of each stage of the life cycle per 1000 *Asterionella* cells. The diamonds, squares and circles mark the dates on which observations were made. % *P*, the percentage of the *Asterionella* cells infected by *Rhizophidium*, is given above the vertical lines marking the dates on which estimations were made (above graph). The dates are also given along the base of the figure.

were constantly aerated at about 17° C. and illuminated by a 1000 W. lamp for 8 hr. a day. After one or two days, all the samples were examined in the usual way and the results are given in Table 1, together with analyses of the original samples and a fresh collection taken on or near the day when the experimental samples were analysed. In all cases, the percentage of infective cells (zoospores and zoosporangia) decreased more in the unaerated samples than in the aerated samples. The decrease in the number of zoospores (per 1000 *Asterionella* cells) is most striking, while the number of sporangia either decreased or increased. It seems that, under artificial conditions, some of the zoospores, already settled on the *Asterionella* cells, are able to develop into sporangia, but the zoospores of the next generation fail to infect further cells and it is this stage of the life history which is most susceptible to changes in environmental conditions.

Table 1. *Laboratory experiments on* Rhizophidium planktonicum *from Esthwaite Water* (see p. 252)

	Exp. 1				Exp. 2			
Date	1. i. 47	3. i. 47	3. i. 47	3. i. 47	9. i. 47	10. i. 47	10. i. 47	11. i. 47
	From lake	Sample of 1. i. 47 after 2 days in laboratory		From lake	From lake	Sample of 9. i. 47 after 1 day in laboratory		From lake
		no aeration	aeration			no aeration	aeration	
Percentage of *Asterionella* cells bearing live *Rhizophidium* cells	16·7	4	12·2	29	31	22	25	34
No. of *Rhizophidium* cells per 1000 *Asterionella* cells								
A. Zoospores	143	17	25	206	150	48	53	142
B. Living sporangia	24	11	95	94	172	156	183	166
C. Empty sporangia	23	57	42	45	82	155	100	166
D. Resting spores	2	2	—	6	7	7	6	4

The failure of *Rhizophidium* to multiply in the laboratory is contrary to the behaviour of most chytridiaceous fungi parasitizing bottom-living or epiphytic algae, particularly those from the mud surface in ponds and *Sphagnum* bogs. In our experience, when these species are removed to the laboratory, they usually increase in numbers until the algal population is almost completely destroyed.

It appears that *Rhizophidium planktonicum* is highly susceptible to changes in environmental conditions and its sudden bursts of activity may well be connected with this. Prof. C. T. Ingold (personal communication) also noted that the planktonic chytrid *Endocoenobium Eudorinae* Ingold soon died after it was removed to the laboratory. However, this susceptibility to changes in conditions seems to be of specific rather than universal application to planktonic chytrids, since de Wildeman (1900, 1931) states that *Rhizophidium Schroeteri* de Wildeman can easily be cultured on *Asterionella* in the laboratory. Sparrow (1943, pp. 65–8) has discussed chytrid epidemics and their possible causes but these are only hypothetical and no successful experimental work has been done.

H. EXTERNAL FACTORS AND THE ONSET AND COURSE OF EPIDEMICS OF *RHIZOPHIDIUM PLANKTONICUM* IN NATURE

No single environmental factor or groups of factors can be demonstrated to determine the onset of an epidemic. The following have been considered with inconclusive results.

Temperature. *Rhizophidium* is present throughout almost the whole period when appreciable numbers of *Asterionella* are available for examination. In 1947, zoospores and sporangia were present under ice in all four bodies of water. In 1946 and 1947, epidemics, with infection reaching 28% of the *Asterionella* cells, occurred under ice in Blelham Tarn, while, in the autumn of 1946, 37% infection was recorded with a surface water temperature of 14° C. and a bottom temperature of 9·2° C.

Light. The same remarks apply here, though epidemic conditions have only once (p. 250) occurred in the period of longest and strongest illumination. Nevertheless, at this period, the end of the spring pulse of *Asterionella*, *Rhizophidium* is always present in large numbers if at low percentage infection (Figs. 6–8). Occasional observations on *Asterionella*, from below the depth receiving 1% of the surface illumination, have not shown any increase or decrease in the numbers of *Rhizophidium* with increasing depth.

Lake level. The occurrence of *Rhizophidium* epidemics is often synchronous with flood conditions (cf. pp. 245, 248–9), but this is not always the case (Esthwaite Water, pp. 243, 245, Figs. 1, 2), nor does the percentage infection during the course of an epidemic show any correlation with the rise and fall of water level.

Dissolved substances. All but one of the epidemics occurred when the concentrations of nitrate, silica and phosphate were high or rising. The same cannot be said of dissolved organic matter on which we have little data. Epidemics can also occur (e.g. Blelham Tarn, autumn 1946, Fig. 4; Esthwaite Water, spring 1947, Fig. 7) when the concentrations of dissolved inorganic matter are low and before the disappearance of thermal stratification. In Blelham Tarn, 1946, the concentrations were rising. All stages in the life cycle, with the exception of resting spores, are present in considerable numbers at the end of the spring pulse of *Asterionella*, when inorganic salts are present in low concentrations and dissolved organic matter is rising. One epidemic (p. 250) occurred at this time. Nothing is know of the actual organic compounds which occur in these waters. The oxygen content of these waters is always high in the upper layers; for most of the period it is close to saturation point.

It is noteworthy, however, that, of the four bodies of water examined, in only the two more eutrophic (Esthwaite Water and Blelham Tarn) did clear-cut epidemics occur. In Windermere south basin there were marked increases in infection, but parasitism never reached 20% of the *Asterionella* cells. In Windermere north basin, the most oligotrophic water, though no less rich in *Asterionella*, no epidemics occurred. *Rhizophidium* occurs in other lakes (p. 251), but only in Loweswater has an epidemic been observed. Loweswater, too, is one of the most eutrophic of the English Lake District lakes.

Antibiotics. Nothing is known about such substances, but it seems unlikely that they are of importance in view of the general occurrence of *Rhizophidium* when appreciable numbers of *Asterionella* are present.

Abundance of Asterionella. This can also be ruled out as a factor, except in so far as *Rhizophidium* appears to occur only sparingly when *Asterionella* is very rare. Yet even this is not certain owing to the impossibility of making accurate estimations at these times.

We have never observed the type of infection described by Wesenberg-Lund (1905, pp. 435–6). Nor do the numbers of host and parasite show the types of relationship described for animals (in particular insects) by DeBach & Smith (1947) except possible 'superparasitism' (DeBach & Smith, 1947, p. 295) which has not been studied. Resting-spore formation is not connected with 'superparasitism'.

It is reasonable to expect that, the more *Asterionella* cells present, the greater the likelihood of zoospores reaching a new host. Since the diameter of a zoospore is only about 3μ it must be largely at the mercy of the water movements even under quiet weather conditions. Its motility is only likely to be of biological advantage when a few microns from the host cell. As to what degree it is advantageous, even at such distances, depends on the scale of turbulent water movements which is not known. If turbulence is on the same scale as at greater distances, then any attractant produced by *Asterionella* would be too regularly diffused in the water, even at such short distances from the cell, to be of any directional aid to the zoospore. In Blelham Tarn in 1946, an epidemic began when there were only 23 *Asterionella* cells per ml. (Fig. 4) and in Esthwaite Water in 1947 when there were 70 cells per ml. Yet only one epidemic (Fig. 7) occurred at the spring maximum of *Asterionella*, when there were from 4000 to 12,000 cells per ml. Nor do conditions unfavourable to the growth of *Asterionella* appear to favour the development of *Rhizophidium* for, again, all but this one epidemic occurred during periods when the diatom numbers were increasing or the cells, judged by appearance and number per colony, were in good physiological condition.

The absence of epidemics during the spring-growth period might be due to the relatively faster growth of *Asterionella*. In general, in this period, the percentage parasitism, though low, remains fairly constant, so that the numbers of *Rhizophidium* rise as those of *Asterionella* do. However, in severe epidemics, at least, the increase rate of the *Rhizophidium* population is greater than that of *Asterionella* at any period (e.g. Figs. 1, 2 and 4).

Predators. Nothing is known of any organisms ingesting the zoospores or attached stages of *Rhizophidium*. There is little doubt that protozoa, ciliates, rotifers and Crustacea could ingest the zoospores since they are known to ingest alga of larger size (cf. Smith, 1937; Vetter, 1937; Pennington, 1941; Margalef, 1945).

Vorticella spp. and small Protomastiginae such as *Salpingoeca* commonly occur on *Asterionella*, but there is no evidence that they protect the cells from infection. *Rhizophidium* occurs on *Asterionella* cells bearing such organisms, nor do these organisms show any marked increase or decrease during epidemics.

Resting Spores. The usual course of epidemics (p. 252) points to their being controlled by some factor or factors other than the above. This applies particularly to the increasing abundance of resting spores, generally seen in the later stages of an epidemic. The zoospores resulting from them differ from those from sporangia in that they are the products of a sexually formed body (p. 258). It might be that the first few generations produced from the zygotes are particularly vigorous. If this were so, then the external factors causing germination of the zygote would be the cause of the onset of epidemics. It is, therefore, desirable to attempt to germinate the zygotes.

I. TAXONOMY OF *RHIZOPHIDIUM PLANKTONICUM* n.sp.

By HILDA M. CANTER

Rhizophidium planktonicum (Figs. 10, 11) appears to be restricted to the diatom *Asterionella.* Spherical sporangia, which may be referable to this fungus, have occasionally been observed on *Tabellaria fenestrata* (Lyngbye) Kützing var. *asterionelloides* Grünow in Windermere south basin.

The infection tube of the zoospore in some specimens apparently penetrates immediately the diatom wall or girdle region, whereas in others a short germ tube is produced on the outside before entry of the host cell is effected. This extra-matrical portion of the germ tube may be straight, forming a short stalk to the mature sporangium (Fig. 11 G), or curl round the alga like a hook (Fig. 11 C), so that the sporangium appears to be sessile. The intramatrical rhizoidal system, when visible, consists of a short, unbranched or often bifid thread which does not taper (Fig. 11 J).

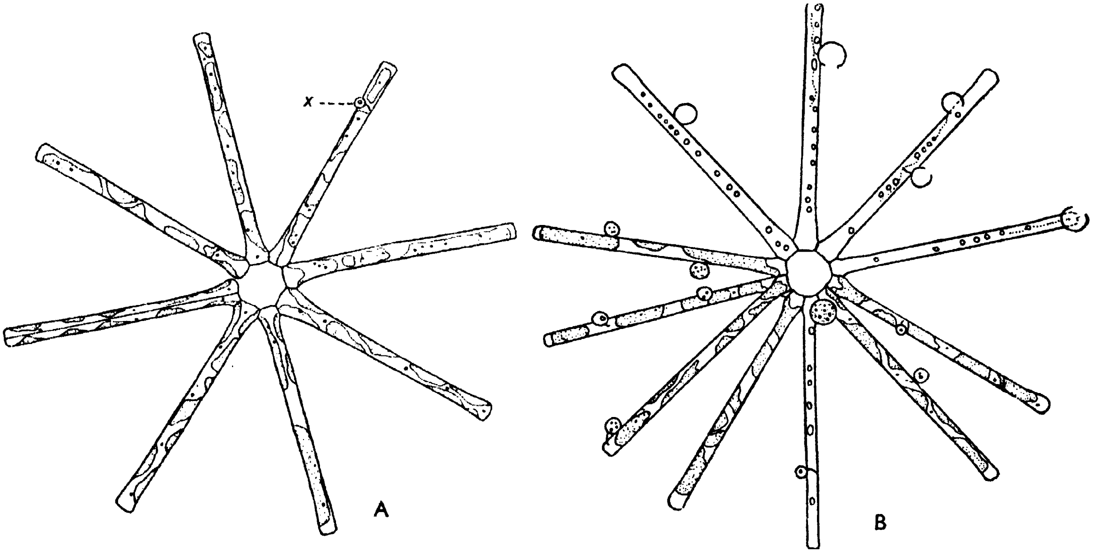

Fig. 10. *Rhizophidium planktonicum.* A, healthy *Asterionella* colony with well-developed chromatophores and encysted zoospore of *Rhizophidium* (*x*). B, heavily parasitized *Asterionella* colony, with more or less disorganized cell contents and *Rhizophidium* sporangia (alive or dehisced). A, B, × 500.

The mature sporangia are spherical $4{\cdot}5$–$9{\cdot}3\,\mu$ in diameter and contain from 4 to 15 oil globules, according to the size of the sporangium, each indicating the position of a zoospore. On dehiscence, a papilla is never formed but a broad apical or lateral pore is developed by deliquescence of the sporangial wall, through which the contents emerge to form a mass just outside the sporangium. After about a minute the zoospores are delimited and those in the more distal region of the mass swim away, the others following almost immediately. The spherical zoospores 3–$3{\cdot}7\,\mu$ in diameter, have a large posterior oil globule, which may be slightly lateral, and a long posterior flagellum. The sporangium wall does not collapse after dehiscence.

Resting spores are formed by a sexual process identical to that found in *Rhizophidium goniosporum* Scherffel and *R. fallax* Scherffel (Scherffel, 1925). The male cell (essentially an encysted zoospore) is attached directly to the female thallus (resembling a young zoosporangium with much oil (Fig. 11 K, L)). When young, the female thallus has a thin wall and one or more large oil globules, but after fusion the wall thickens and the oil becomes more dispersed forming several smaller globules (Fig. 11 N, O); a single large globule is never present in the mature zygote. The spherical resting spore, 4–7μ in diameter, always has a smooth wall which, being highly refractive appears as a black line under the microscope. The male, 2–3μ in diameter, remains as an empty appendage to the resting spore (Fig. 11 N, O). Occasionally two males were seen attached to a female thallus (Fig. 11 L). Germination of the resting spore was not observed.

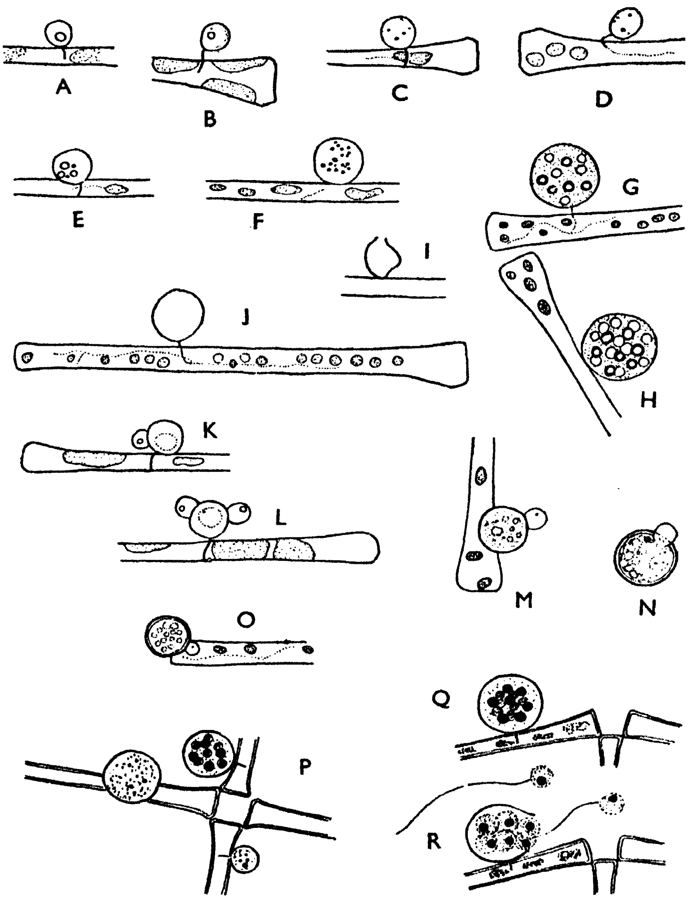

Fig. 11. *Rhizophidium planktonicum.* A–F, early stages in the development of sporangia; G, H, mature sporangia; I, J, empty sporangia, rhizoidal system visible in J; K, L, young female thalli with adherent male cells; M, immature, and N, O, mature, zygotes (resting spores) with empty adherent male cells; P, R, chytridiaceous fungus on *Asterionella* from Kilby Bridge; drawings by Prof. C. T. Ingold. Note progressive disorganization of *Asterionella* chromatophores from A–J and K–O. A–J, L–N, × 1050; K, O, × 800; P–R, × 830.

A chytrid similar to the one described above was found by Prof. C. T. Ingold in March 1941, parasitizing *Asterionella* sp. from Kilby Bridge, Leicestershire, England (Fig. 11 P–R); no resting spores were seen.

Although the type of sexual reproduction resembles closely that found in *Rhizophidium fallax* and *R. goniosporum* (Scherffel, 1925), *R. planktonicum* cannot be identified with either of these species. In *R. fallax* the zoospores emerge individually from the sporangium through one or more minute needle-like openings; in *R. goniosporum* the sporangium has two lateral opposite papillae, and the resting spore is polygonal.

Two parasites have been recorded on *Asterionella*. De Wildeman (1900, 1931) described *Rhizophidium Schroeteri* de Wildeman from the plankton of Lake Zürich, Switzerland. Although resembling *R. planktonicum* in the small size of the sporangium, in the small number of zoospores and in the simplicity of the rhizoidal system it, nevertheless, differs in several respects. A definite hyaline papilla is formed which dissolves at maturity and the zoospores are 1 μ in diameter (cf. *R. planktonicum* 3·4 μ).

More recently Huber-Pestalozzi (1946) records the presence of a small spherical chytrid on *Asterionella formosa* from the Walensee, differing from the British form in the unbranched rhizoid which sometimes ends in a knob-like structure. Neither dehiscence of the sporangium nor resting spores were seen.

Rhizophidium sphaerocarpum (Zopf, 1884) Fischer (1892), *R. constantineani* Saccardo (1905) and *R. Mischococci* Scherffel (1926) all bear some resemblance to *R. planktonicum* but do not exactly correspond. The author is aware that the delimitation of species on morphological grounds is very difficult in *Rhizophidium* but, because of its common and constant occurrence on *Asterionella* and its very characteristic resting spore, it would seem best to place this fungus in a distinct species and consequently the binomial *R. planktonicum* is proposed, taking its name from the habitat in which it so characteristically occurs.

Rhizophidium planktonicum n.sp.

Thallus epibiotic sessile or stalked, sporangium spherical, 4·5–9·3 μ in diameter, with 4–15 zoospores. Zoospores spherical 3–3·7 μ in diameter, uniguttulate, posteriorly uniflagellate, emerging in a mass on gelatinization of the apex of the sporangium wall. Intramatrical rhizoid unbranched or once branched, not tapering. Resting spores spherical 4–7 μ in diameter, wall smooth, the content with numerous small oil globules; arising from fusion of the contents of a small male with a larger female cell, the former remaining as an appendage to the mature resting spore. Germination unknown.

Parasitic on *Asterionella formosa* Hass. in Windermere, Esthwaite Water and Blelham Tarn, near Wray Castle, Ambleside, England.

Rhizophidium planktonicum sp.nov.*

Thallus epibioticus, sessilis vel pedicellatus. Sporangium globosum, 4·5–9·3 μ diametro, zoosporis 4–15 inclusis. Zoosporae sphaericae, 3–3·7 μ diam., uniguttulatae, postice uniflagellatae, in massa per apicem sporangii gelatinisatum emergentes. Rhizoideum intramatricale, aut simplex aut ramulo singulo praeditum, aequale. Sporae perdurantes sphaericae, 4–7 μ diametro, pluriguttulatae, membrana laevi, per conjugium cellulae masculae parvae cum cellula feminea majore ortae, illa ut appendicula sporae maturae persistente. Germinatio nondum visa.

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* My thanks are due to Miss E. M. Wakefield of Kew for the Latin translation.

Hab. in *Asterionellam formosam* Hass. parasiticus. Windermere, Esthwaite Water et Blelham Tarn, prope Wray Castle, Anglia.

J. SUMMARY

The chytridiaceous fungus *Rhizophidium planktonicum*, a new species, is parasitic on the plankton diatom *Asterionella formosa* Hass. in Windermere, Esthwaite Water and Blelham Tarn, lakes in the English Lake District.

The parasite is almost always present when the host is. For a large part of the year, the frequency of the parasite is too low to reduce appreciably the numbers of *Asterionella*.

At certain times, particularly in autumn and winter, the numbers of *Rhizophidium* increase rapidly causing an epidemic. The numbers of live *Asterionella* cells are reduced, while the ratio of dead to live cells rises. In all epidemics the average number of live cells per *Asterionella* colony decreases.

The *Rhizophidium* population passes through a series of characteristic phases during an epidemic.

It has not been possible to grow *Rhizophidium* under artificial conditions nor have any single environmental factors or groups of factors been demonstrated as determining the onset of epidemics.

The authors' thanks are due to Mr H. C. Gilson and Prof. C. T. Ingold for advice and criticism, and to members of the scientific and laboratory staff of the Freshwater Biological Association, especially Mr D. Gawen, for assistance, particularly in sampling.

REFERENCES

CHU, S. P. (1942). The influence of the mineral composition of the medium on the growth of planktonic algae. *J. Ecol.* 30, 284–325.

DEBACH, P. & SMITH, H. S. (1947). Effects of parasite population density on rate of change of host and parasite populations. *Ecology*, **28**, 290–8.

FISCHER, A. (1892). Phycomycetes. Die Pilze Deutschlands usw. Rabenhorst, *Kryptogamen-Fl.* **1**. Leipzig.

GARDINER, A. (1940–1). Fluctuations in the number of cells per colony of the diatom *Asterionella formosa*. *Proc. Linn. Soc. Lond.* 153rd Sess., 160.

HUBER-PESTALOZZI, G. (1946). Der Walensee und sein Plankton. *Z. Hydrol.* **10**, 1–200.

HUSTEDT, F. (1938). Bacillariophyta. Rabenhorst, *Kryptogamen-Fl.* **7**, Teil 2.

MARGALEF, R. (1945). Observaciones sobre el régimen alimenticio de varios pequeños animales de agua dulce. *Inst. Biol. Apl. Secc. Fisiol. anim. Barcelona*, **1**, 245–50.

MILL, H. R. (1895). Bathymetrical survey of the English lakes. *Geogr. J.* **6**, 46–73, 135–66.

MORTIMER, C. H. (1941–2). The exchange of dissolved substances between mud and water in lakes. *J. Ecol.* **29**, 280–329; **30**, 147–201.

PEARSALL, W. H., GARDINER, A. C. & GREENSHIELDS, F. (1946). Freshwater biology and water supply in Britain. *Sci. Publ. Freshwater Biol. Ass. Brit. Emp.* **11**.

PENNINGTON, W. (1941). The control of the numbers of freshwater phytoplankton by small invertebrate animals. *J. Ecol.* **29**, 204–11.

REYNOLDS, N. (1940). Seasonal variations in *Staurastrum paradoxum* Meyen. *New Phytol.* **39**, 86–9.

SACCARDO, D. & P. A. (1905). *Sylloge Fungorum*, **17**. Padua.

SCHERFFEL, A. (1925). Zur Sexualität der Chytridineen (Beiträge zur Kenntnis der Chytridineen, Teil I). *Arch. Protistenk.* **53**, 1–58.

SCHERFFEL, A. (1926). Einiges über neue oder ungenügend bekannte Chytridineen (Beiträge zur Kenntnis der Chytridineen, Teil II). *Arch. Protistenk.* **54**, 167–260.

SMITH, M. W. (1937). Notes on the food of *Daphnia pulex* (de Geer) in fertilized water. *Trans. Amer. Fish. Soc.* (1936), pp. 287–90.

SPARROW, F. K. (1943). *Aquatic Phycomycetes*. Ann Arbor.

UTERMÖHL, H. (1931). Über das umgekehrte Mikroskop. *Arch. Hydrobiol. Plankt.* **22**, 643–5.

VETTER, H. (1937). Bemerkungen über das Phytoplankton und seine Beziehungen zur Ernährung des Crustaceenplankton im Schussaltwasser und im Bühelweiher. *Schr. Ver. Ges. Bodensees u. s. Umgeb.* **64**, 5–15.

WELCH, P. S. (1935). *Limnology*. New York.

WESENBERG-LUND, C. (1905). A comparative study of the lakes of Scotland and Denmark. *Proc. Roy. Soc. Edinb.* **25**, 401–48.

WESTON, W. H. (1941). The role of the aquatic fungi in hydrobiology. *A Symposium on Hydrobiology*, pp. 129–51. Madison.

WILDEMAN, E. DE (1900). Observations sur quelques Chytridinées nouvelles ou peu connues. *Mém. Herb. Boissier*, pp. 1–2.

WILDEMAN, E. DE (1931). Sur quelques Chytridinées parasites d'algues. *Bull. Acad. Belg. Cl. Sci.* **5**, 281–98.

ZOPF, W. (1884). Zur Kenntnis der Phycomyceten. I. Zur Morphologie und Biologie der Ancylisteen und Chytridiaceen. *Nova Acta Leop. Carol.* **47**, 143–236.

LATE-GLACIAL DEPOSITS IN BERWICKSHIRE

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At a late stage in the glacial period south-west Berwickshire was covered by ice which formed in the Cheviot and Lowther Hills and moved thence in a north-easterly direction (Pringle, 1935). As a result this part of the county is to-day covered by undulating ridges of drift, which lie parallel with the direction of ice movement and enclose numerous small basins. Some bosses of rock protrude through the drift. In the basins late-glacial and post-glacial deposits have accumulated, and a paper published seventy years ago (Smith, 1873) gives a description of the deposits in one of the basins, Whitrig Bog, which lies 6½ miles west of Kelso at an altitude of 500 ft.

The description is as follows: 'Whitrig Bog has been extensively cut into from its western extremity, and a large brick and tile works has been in operation there for many years, making use of the brick clay which lies below the shell marl of the peat bog. On the north edge of the bog, there is a partial bed of sand about 3 ft. in greatest depth; beyond this, is found the great bed of peat, averaging about 10 ft. in thickness, and containing portions of trees; one, which I examined, was of birch. Below the peat there is an extensive bed of shell marl; for 3 ft. in thickness you have pure marl, then over a part at least of the bog there is a bed of blue clay about 3 ft. in thickness, and below this again, there are other 3 ft. of marl. Under the marl is a bed of brick clay, averaging 6 ft. in thickness, and underneath this clay you come at last to the hard till, or boulder clay.'

In many enclosed basins in Denmark (Iversen, 1947) and in Ireland (Jessen & Farrington, 1938; Mitchell, 1941) similar sequences of deposits have been described. These are interpreted as recording (*a*) the moulding of the basin by an ice-sheet, (*b*) the deposition as the ice withdrew of a stone-free clay containing the remains of arctic plants, (*c*) the accumulation during the more genial Allerød period of a mud containing the remains of some temperate plants, (*d*) the deposition of sand and clay containing the remains of arctic plants all carried by frost action out into the basin from the surrounding slopes, and (*e*) the accumulation of mud and peat in the post-glacial period.

In September of this year I paid a short visit to Whitrig Bog with Dr H. Godwin, F.R.S., and at the north-west margin of the bog we were fortunate enough to discover in the wall of a long-abandoned brick pit an exposure showing marl resting on red clay. Leaves of *Salix herbacea* and *S. reticulata* were common in the upper layers of the clay. We dug a small pit in the clay which proved to be over 2 ft. thick and to rest on a lower marl. Samples for washing and for microscopic examination were collected from the base of the upper marl, the top of the clay, the clay in general and from the top of the lower marl, and Table 1 shows the macroscopic and microscopic fossils identified in the samples. I am indebted to Mr J. B. Duncan of Berwick-on-Tweed for the identification of the mosses and to Mr A. W. Stelfox of the National Museum of Ireland for the identification of the molluscs.

As only a few samples had been collected for microscopic examination it was felt that they did not merit the prolonged study that would have been necessary (on account of the sparseness of the contained pollen) to count statistically reliable numbers of pollen grains. But the limited examination they received indicated that pollen grains were more numerous in the marls than in the clay, and that the proportion of arboreal-pollen to non-arboreal-pollen was higher in the marls than in the clay. *Pinus* was more common in the clay, to which *Artemisia* was virtually confined, while *Empetrum* was more common in the marls than in the clay. Remains of mosses, including some large-leaved forms, were common in the clay.

Table 1. *Plant and animal fossils from Whitrig Bog, Berwickshire*

Fossils	Base of upper marl	Top of clay	Clay in general	Top of lower marl
Armeria cf. *vulgaris*	m	M	M	.
Artemisia sp.	m	m	m	.
Betula nana	.	.	M	M
B. pubescens	.	.	.	M
Betula sp.	m	m	m	m
Carex sp.	M	M	.	.
Caryophyllaceae	m	m	m, M	m
Ceratophyllum sp.	m, M	.	.	.
Cyperaceae	m	m	m	m
Empetrum sp.	m	m	m	m
Gramineae	m	m	m	m
Helianthemum sp.	m	.	.	m
Myriophyllum sp.	M	M	.	M
Pinus sp.	m	m	m	m
Potamogeton filiformis	M	.	.	.
Potamogeton cf. *praelongus*	M	M	.	.
Ranunculus aquatilis	M	M	.	.
Rumex acetosella	M	M	.	.
Rumex sp.	m	m	m	.
Salix herbacea	.	M	M	.
S. reticulata	.	M	.	.
Salix sp.	m	m	m	m
Thalictrum alpinum	.	M	.	.
Umbelliferae	.	.	.	m
Characeae	M	M	M	M
Aulacomnium palustre	.	M	.	.
Fontinalis antipyretica	.	.	M	.
Hypnum fluitans	.	M	.	.
H. giganteum	.	M	.	.
H. sarmentosum	.	M	.	.
H. scorpioides	.	M	M	.
H. turgescens	.	M	M	.
Hypnum sp.	.	M	.	.
Selaginella selaginoides	m	.	m	.
Botryococcus sp.	m	m	m	m
Cosmarium sp.	m	m	.	m
Pediastrum sp.	m	m	m	m
Limnaea perager	M	.	.	.
Pisidium casertanum	M	.	.	.
P. obtusalis	M	.	.	.
Planorbis crista	M	.	.	.
P. laevis	M	.	.	.
Valvata piscinalis	M	.	.	.
Coleoptera	M	M	M	M
Cladocera sp.	m	m	.	m
Daphnia sp.	.	M	.	.
Ostracoda	M	.	.	.
Cristatella	M	M	.	.

M = macroscopic fossils (e.g. fruits, seeds, stems, leaves, shells, carapaces); m = microscopic fossils (e.g. pollen, spores).

It seems certain that this layer of red clay lying between an upper and a lower marl must be the equivalent of the blue clay noted by Smith in a similar position. As the bog lies in a drift-enclosed basin it is reasonable to suppose that the clay is a late-glacial solifluction deposit, and that while it was being formed *Salix herbacea*, *S. reticulata* and *Betula nana* were growing on the surrounding slopes. The lower marl containing fruits of *B. pubescens* suggests more genial conditions, and this deposit probably belongs to the Allerød period. The presence of the pollens of *Artemisia*, *Empetrum* and *Helianthemum* supports the view that the clay and lower marl are late-glacial deposits. The upper marl and the peat belong to the post-glacial period.

If this interpretation is accepted the late-glacial sequence of events in southern Scotland would seem to have been closely parallel to that in Denmark and in Ireland. But in view of the record (Lewis, 1905) of a layer with much *Empetrum nigrum*, *Salix herbacea* and *S. reticulata* overlying peat rich in *Eriophorum vaginatum* in some mountain bogs in the western part of the Southern Uplands some caution is necessary until a full investigation of the stratigraphy of Whitrig Bog is carried out. The southern margin of the bog is partly bounded by a cliff of igneous rock, and as the cliff probably carried a rich phanerogamic flora in late-glacial times (as some cliffs in the Highlands do to-day) excavations at the foot of the cliff might produce many additions to the limited flora noted here.

REFERENCES

IVERSEN, J. (1947). Plantevaekst, Dyreliv og Klima i det Senglaciale Danmark. *Geol. Fören. Stockh. Förh.* **69**, 1.

JESSEN, K. & FARRINGTON, A. (1938). The Bogs at Ballybetagh, near Dublin, with remarks on late-glacial conditions in Ireland. *Proc. Roy. Irish Acad.* **44** B, 10.

LEWIS, F. J. (1905). The plant remains in the Scottish peat mosses. Part I. The Southern Uplands. *Trans. Roy. Soc. Edinb.* **41**, 28.

MITCHELL, G. F. (1941). Studies in Irish quaternary deposits. 2. Some lacustrine deposits near Ratoath, Co. Meath. *Proc. Roy. Irish Acad.* **46** B, 13.

PRINGLE, J. (1935). *British Regional Geology; The South of Scotland.* Edinburgh.

SMITH, J. A. (1873). Notice of the discovery of remains of the Elk (*Cervus Alces* Linn., *Alces Malchis* Gray), in Berwickshire; with notes of its occurrence in the British Islands, more particularly in Scotland, etc. *Proc. Soc. Ant. Scot.* **9**, 297.

DIFFUSION AND ABSORPTION IN DISKS OF PLANT TISSUE

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(With 1 figure in the text)

Most investigations of cells of higher organisms are carried out with cells aggregated in the form either of whole organs or of slices of tissue: to deduce the behaviour of cells from the results of such experiments is one of the major physiological problems. It is complicated if the cells forming the aggregate differ inherently in the physiological activity investigated. Even when the aggregate consists of cells which are, in themselves, as alike as possible, there remains the problem of deducing the environment of the cell from the data of the environment of the tissue. The activity of the cells will cause a gradient of concentrations of substances consumed and produced, with the consequence that the environment of the cells may be appreciably different from that portion of the environment which is sampled. Cells, otherwise alike, may vary in the resistance they offer to the passage of substances into them, with resulting variations in the differences between the concentrations of substances at the seats of reaction within the cell and the concentrations in the immediate surroundings.

It is the fundamental problem of supply and consumption, or production and removal, and the effect of these processes on the concentration of substances determining the rates of the consumption or production. Here it will be considered in relation to respiration and salt accumulation by slices of plant tissue; carrot roots in particular.

Disks of tissue cut from plant organs such as carrot root and potato tuber are frequently used in experiments on respiration and on absorption of substances from solution. In the latter, there has been no detailed consideration of the change of concentration of the substances with increasing distance from the surface of the disk. Any such change must be taken into account in an attempt to deduce information concerning the activity of the cells from the observed activity of the disk. The substances in solution which must be considered are not only those whose absorption is being studied but also oxygen and carbon dioxide, the concentration of which may affect the metabolic processes concerned with absorption as well as respiration. The inter-relation of processes such as salt absorption and respiration must also be borne in mind.

In the past many investigators have tended to neglect, or have been content with vague references to, consumption and production or diffusion according to whether their chief interest has been resistance to diffusion or absorption by the tissue.

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* The experimental work was done during the tenure, from 1936 to 1938, by R.N.R. of a Scholarship of the Royal Commission for the Exhibition of 1851.

GENERAL STATEMENT OF THE PROBLEM

Let us consider a disk, relatively large compared with its thickness so that effects due to the edge can be neglected and, in the first place, let the disk be uniform with respect to its resistance to passage of solutes. Then

$$\frac{\partial c}{\partial t}=K\frac{\partial^2 c}{\partial x^2}-p, \tag{1}$$

where c is the concentration in a plane parallel to the surface, the x axis is normal to this plane, p is the rate of consumption (or $-p$ the rate of production) per unit volume, K is the coefficient of diffusion, and t is the time. Disks of plant material with the intercellular spaces injected with water may approach this ideal state for substances such as oxygen and carbon dioxide. For larger molecules such as sugar, and for electrolytes, the disks behave as if the movement of the substances was restricted mainly to the media around the vacuoles. Such a system can be treated as if it consisted of a phase a, occupying a fraction q of the total volume, in which the solute moves and from which it is removed by consumption by or passage into the other phase.* Then the equation for c_a, the concentration in a, is

$$\frac{\partial c_a}{\partial t}=\frac{1}{q}\left(K\frac{\partial^2 c_a}{\partial x^2}-p\right), \tag{2}$$

where K and p refer to the disk as a whole. As to whether q is the actual ratio of the volume of the phase a to the total volume and other details need not concern us while we are concerned with the broad picture.

When considering states where $\partial c/\partial t$ is not zero the difference between equations (1) and (2) is of importance. We shall be considering states in which, although the concentration in a given plane is changing with time, yet, the value of $\partial c/\partial t$ is relatively so small that it can be neglected. Then q does not appear in the equation and we shall use c for the concentration of the solute in the phase in which it moves freely, and

$$K\frac{d^2 c}{dx^2}-p=0. \tag{3}$$

There are two special cases which require consideration: first, where the concentrations at the two surfaces of the disk are different; and second, where they are the same. Experiments, such as those of Steward (1930), designed to measure the diffusivity of solutes, are examples of the first kind and these we shall call 'diffusion' experiments. Most of the experiments on absorption by disks are of the second kind and these we shall call 'absorption' experiments. As we have already pointed out diffusivity *and* absorption are factors involved in both kinds.

Before we can solve equation (3) we must know the relation between p and c. Unfortunately, this relation for substances of great interest does not appear to be of a form giving a simple solution to the equation. There is the added complication that the production of carbon dioxide and the absorption of salts depend upon the concentration of another substance, oxygen, and, further, in some circumstances it appears that the inherent activity of the cells, and hence p, changes with distance from the surface which has recently been affected by cutting.

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* We are not concerned here with what happens to the substances which are removed, nor with the origin of the substances which are produced.

Since, for many substances, the relation between p and c approaches close to proportionality for low concentrations and to independence for high values we shall consider in detail these two special cases: $p=hc$ and p a constant. The complications we have mentioned must be borne in mind in applying the solutions of the equation to the experimental results.

CONSUMPTION BY CELLS INDEPENDENT OF CONCENTRATION

Taking first $\frac{d^2c}{dx^2}=\frac{p}{K}$, where p is a constant, we have

$$\left.\begin{aligned} \frac{dc}{dx} &= \frac{px}{K}+A, \\ c &= \frac{px^2}{2K}+Ax+B. \end{aligned}\right\} \tag{4}$$

Let us consider a disk of thickness, l, with c_1 the lower concentration, at $x=0$, and c_2, the higher, at $x=l$. Then $B=c_1$ and L, the loss per cm.^{2} of the substance from the disk at $x=0$, will be KA, and U, the uptake per cm.^{2} at $x=l$, will be $KA+pl$, and pl is the rate of absorption per cm.^{2} of disk.

Since
$$c_2=\frac{pl^2}{2K}+Al+B=\frac{(U-L)\,l}{2K}+\frac{Ll}{K}+c_1,$$

$$\frac{c_2-c_1}{l}=\frac{U+L}{2K}. \tag{5}$$

So from measurements of uptake and loss for known values of c_1 and c_2 we can determine the value of K, the coefficient of diffusion.

In the calculations of the coefficient of diffusion of sugar and potassium phosphate in disks of beet and potato Steward (1930) neglects the absorption, that is, he assumes $p=0$ and $U=L$. In those experiments where both uptake and loss were measured, inspection of the results reveals that this neglect is not justified. In those experiments where the cells were plasmolysed this point is not so important but, where the cells are more nearly normal, e.g. Exp. 2, Table 3, and Exp. 2, Table 5, U exceeded L by 68 and 97 % respectively. Hence, if p is a constant, Steward's estimates of the coefficient of diffusion should be increased by 34 and 48 %. We shall consider these results again in a later section.

Diffusivity of carbon dioxide in tissue.

Our experiments on the rate of production of carbon dioxide by disks of carrot root in aerated solution show that the rate per cm.^{3} of tissue, after allowing for the cut surface cells, is independent of disk thickness from 0·05 to 0·2 cm. Hence it seems reasonable to assume that over this range p for carbon dioxide production, and probably for oxygen consumption, is a constant. In using our equation to ascertain the value of the coefficient of diffusion of carbon dioxide in the tissue, we are faced with the complication that the area of the disk is greater than the area exposed to the solution on either side, by the amount covered by the flange holding the disk in position between the two solutions of different concentration of carbon dioxide. An approximate solution to the difficulty is obtained by adding to the area exposed to the solution, a fraction (for our experiments about a half) of the area covered by the flange, and so getting a corrected area (see Appendix A).

The method of determining carbon dioxide was not accurate enough to measure the uptake (a small precentage change in the concentration of carbon dioxide). Consequently, the rate of production of carbon dioxide by the disk, R, was measured before and after the 'diffusion' experiment and, having determined L, the value of U was determined from $L-R$. Experiments at 21° C. with carrot tissue cut tangentially in the phloem region and radially in the xylem region gave values for the coefficient of diffusion of the same order, ranging from $1{\cdot}4$ to $2{\cdot}2 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ with a mean of $1{\cdot}7 \times 10^{-5}$. These figures are in terms of the concentration of carbon dioxide in air in equilibrium with the solution, not of the concentration in the solution. Since increase of temperature reduces the solubility of carbon dioxide in water to practically the same extent as it increases the real coefficient of diffusion, temperature has little effect on the value of K for carbon dioxide in water in the units we have used. Comparison with the coefficient of diffusion in water at 15° C. shows that the tissue offers no more resistance than does water which contributes about 80 % of the weight of the tissue.

Since the coefficient of diffusion is about 10^4 times greater in air than in water there is the possibility that the value of K for the tissue may be markedly affected by the amount of the intercellular spaces occupied by air. From experiments on the change in weight of disks while suspended in water, we can conclude that less than 3 % of the volume of the disk is occupied by gas phase when the disk is in aerated water. When aeration is stopped the intercellular spaces become injected with water and the weight of the immersed disk is increased correspondingly. The disposition of these air spaces as well as their dimensions is of importance. If arranged as tubes continuous from face to face of the disk they would have the maximum effect on the passage of the carbon dioxide. Actually they are distributed throughout the tissue at the corners of the cells. An idea of their effect can be obtained by considering the gas phase as small cubes at the corners of otherwise cubical cells, both sets of cubes oriented with faces parallel to the face of the disk. If the small cube has a side a fraction x of that of the large cube, and the coefficient of diffusion in the gas phase is P times that in the non-gaseous phase, K, then the average value for the tissue is

$$K\left[(1-x^2)+\frac{Px^2}{x+P(1-x)}\right].$$

With x at $0{\cdot}35$, that is a gas phase $4{\cdot}3$ % of the total, the value of the coefficient of diffusion for the tissue is only $6{\cdot}6$ % greater than that for the non-gaseous phase. Hence we may conclude that our results give us a fairly accurate result for the coefficient of diffusion of carbon dioxide in the non-gaseous part of the carrot tissue.

Concentration of carbon dioxide and oxygen in the disk

It is difficult to carry out similar experiments for oxygen but, in view of the suggestions which have been made that the centre of disks used in some absorption experiments may be suffering from shortage of oxygen, a knowledge of the coefficient of diffusion of oxygen in the tissue is important. If we make the not unreasonable asumption that the value of the coefficient of diffusion for carbon dioxide in carrot tissue indicates that the non-gaseous phase in all its parts behaves like water in the resistance it offers to the passage of gases such as carbon dioxide and oxygen, which are in the form of undissociated molecules, then we can use the coefficient of diffusion of oxygen in water for that in the tissue ($6{\cdot}5 \times 10^{-7}$ cm.^{2} sec.$^{-1}$).* There is, of course, the possibility that in portions of the tissue the coefficient of diffusion of carbon dioxide is lower than in water and in others, where the carbon dioxide is more soluble than in water, it is higher, and that these portions behave differently to oxygen and carbon dioxide. The solubility of the gas comes into consideration because our values for K are in terms of concentration in air in equilibrium with the solution. The presence of gas in the intercellular spaces has practically the same effect for the passage of oxygen as for carbon dioxide.

Taking the above coefficients of diffusion for oxygen and carbon dioxide and assuming that p is constant for oxygen consumption as well as for carbon dioxide production, we can calculate the concentration (in air in equilibrium) at different distances from the surface of disks in 'absorption' experiments where the concentration of the substance, c_0, is the same at the two surfaces $x=0$ and $x=l$.

Equation (4) becomes

$$c = c_0 - \frac{px(l-x)}{2K}. \tag{6}$$

The concentration of carbon dioxide will rise (because p is negative) as the centre plane of the disk is approached, most rapidly at first, with the gradient falling to zero at the middle. The relation for oxygen will be the inverse since here p is positive. The concentration at the centre, $c_{\frac{1}{2}}$, is given by

$$c_{\frac{1}{2}} = c_0 - \frac{pl^2}{8K}. \tag{7}$$

For carrot disks minus p is about 8×10^{-5} mg. CO_2 per cm.^{3} of tissue per sec. This gives an excess of the concentration of carbon dioxide over that at the surface of 3 cm.^{3}/l.† for a disk of 0·1 cm. thickness, while the deficit of oxygen is 80 cm.^{3}/l. Since the solution around the disk in our experiments has negligible concentration of carbon dioxide and has an equilibrium pressure of oxygen of about 18·5 % the equilibrium pressures for the centre of the disks are about 0·3 % carbon dioxide and 10·5 % oxygen. For the disks of 0·2 cm. the oxygen pressure will drop to 2·5 %. The deficit of oxygen cannot continue to rise with the increase of thickness because eventually the point will be reached where the consumption will be limited by the concentration, that is p will no longer be a constant.

If we granted Steward's assumption that the diffusion of oxygen in tissues is restricted to the same extent as is the diffusion of salts, then K for oxygen in carrot would be $1{\cdot}7 \times 10^{-8}$ cm.^{2} sec.$^{-1}$ Using equation (7), the difference between the concentration of oxygen at the centre of the disk and at the surface is 300 %. Since the concentration at the surface does not exceed 21 %, we must conclude that K cannot be so small while p remains constant.

Steward (1932) found that, with potato disks, the rate of respiration per gramme of tissue increased as the thickness of the disk was reduced below 0·1 cm. and suggested, in explanation, that the respiratory activity of the cells at the centre of the disks of 0·1 cm.

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* Steward (1932) suggests that diffusion of oxygen in plant tissue, as compared with that in water, is reduced to the same extent as is the diffusion of salts and sugar, but he gives no evidence in support of this suggestion.

† Concentrations in air in equilibrium with water.

thickness was reduced by oxygen deficiency. The rate of respiration of disks of 0·1 cm. was practically the same as the figure quoted above for carrot disks and hence, if K for oxygen is the same for both, the concentration of oxygen at the centre of the potato disks should be about 10 %. If the respiration of the cells at the centre were reduced by lowering the oxygen to this level, then one would expect the respiration of the disks to be increased by raising the concentration outside the disks above 21 %. Since an increase to 100 % has no such effect, it seems that an explanation different from that of oxygen deficiency must be sought (see p. 277).

Steward suggests, also, that respiration is highest in a surface layer as a result of injury in cutting, and that the respiration falls linearly on passing through this surface layer towards the centre. This fall presumably represents a fall in activity of the cells, unconnected with any fall of oxygen concentration, since, when this surface layer is passed in thicker disks, the cells nearer the centre are assumed to respire at a rate independent of distance from the surface.

The effect of such a distribution of respiratory activity on the concentration of oxygen and carbon dioxide at the centre can be deduced as follows:

Our equation becomes
$$K\frac{d^2c}{dx^2}=p-ax,$$

where x is measured from the surface.

$$\frac{dc}{dx}=\frac{px}{K}-\frac{ax^2}{2K}+b,$$

$$c=\frac{px^2}{2K}-\frac{ax^3}{6K}+bx+c_0,$$

$$b=\frac{\rho}{2K},$$

where ρ = rate of respiration per cm.^{2} of disk.

$$c_{\frac{1}{2}}-c_0=\frac{pl^2}{8K}-\frac{al^3}{48K}+\frac{\rho l}{4K}.$$

For $l=0{\cdot}0829$ cm., minus $p=10{\cdot}08\times10^{-5}$ mg. CO$_2$/cm.^{3}/sec., minus a is $20{\cdot}4\times10^{-4}$ mg. CO$_2$/cm.^{3}/cm./sec., and ρ is 5·3 mg. CO$_2$/cm.^{2}/sec. This gives an excess of concentration of carbon dioxide at the centre, 1·45 cm.^{3}/l., and a deficit of oxygen of 39 cm.^{3}/l. If the external solution were in equilibrium with 21 % oxygen, that at the centre would be in equilibrium with over 17 %. If the respiration were uniform throughout the disk, giving a value for minus p of $6{\cdot}4\times10^{-5}$ mg. CO$_2$/cm.^{3}/sec., the oxygen at the centre would be a little smaller, 16·5 %.

CONSUMPTION BY CELLS PROPORTIONAL TO CONCENTRATION

We now turn to the case where the consumption is proportional to the concentration, $p=hc$;* h may be called the absorption coefficient. Experiment suggests that, for the absorption of salts by carrot tissue, the justification for such an assumption increases as the concentration is reduced. The solution of the equation

$$\frac{d^2c}{dx^2}=\frac{hc}{K}$$

is

$$c=A\sinh\mu x+B\cosh\mu x, \tag{8}$$

where

$$\mu^2=\frac{h}{K}.$$

For 'diffusion' experiments

$$c=c_1 \text{ at } x=0 \quad\text{and}\quad c=c_2 \text{ at } x=l,$$

then

$$c=\frac{c_1\sinh\mu(l-x)+c_2\sinh\mu x}{\sinh\mu l}. \tag{9}$$

The loss per cm.^{2} to the solution at $x=0$ is

$$K\frac{dc}{dx}=\frac{K\mu}{\sinh\mu l}(c_2-c_1\cosh\mu l)=L,$$

and the uptake from the solution at $x=l$ is

$$\frac{K\mu}{\sinh\mu l}(c_2\cosh\mu l-c_1)=U,$$

hence

$$\frac{U}{L}=\frac{c_2\cosh\mu l-c_1}{c_2-c_1\cosh\mu l},$$

and

$$\cosh\mu l=\frac{Uc_2+Lc_1}{Lc_2+Uc_1}. \tag{10}$$

From this the value of μ can be obtained and used in the following equation to give the value of K, and hence the value of h.

$$K=\frac{U\sinh\mu l}{\mu(c_2\cosh\mu l-c_1)}. \tag{11}$$

For 'absorption' experiments where $c=c_0$ at $x=0$ and $x=l$ the uptake per cm.^{2} is the same for both surfaces and is given by

$$U=\frac{K\mu c_0}{\sinh\mu l}(\cosh\mu l-1). \tag{12}$$

Using the value of K obtained from the results of 'diffusion' experiments the value of μ and hence of h can be obtained.

The rate of absorption per cm.^{3} disk, R, is given by

$$R=\frac{2U}{l}=hc_0\frac{2(\cosh\mu l-1)}{\mu l\sinh\mu l}.$$

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* Although we know little about the factors determining the absorption of solutes by plant cells—to what extent the rate is limited by metabolic activity on the one hand and resistance of the membrane surrounding the cell on the other—yet there is much to be said for expressing the rate of absorption per unit of cell surface as well as per unit of cell volume. The cells of carrot tissue are not very regular, but a rough estimate gives about 1500 cm.^{2} of cell surface per cm.^{3} of tissue. Hence to convert rates per cm.^{3} into rates per cm.^{2} of cell surface the figures should be divided by 1500 giving a unit of dimensions cm. sec.$^{-1}$

At $\mu l = 0{\cdot}1$, R is very nearly equal to hc_0 because, throughout the disk, c is practically equal to c_0. As μl is increased, either by increasing h relative to K or by increasing l, R falls appreciably below hc_0 and has dropped to less than a third of this value when $\mu l = 4$. This reflects the steeper fall of c as the centre of the disk is approached.

Diffusivity of salts in tissue and absorption coefficient

(1) *'Diffusion' experiments*

Diffusion experiments were carried out with carrot disks about 0·1 cm. thick in potassium chloride. The uptake and loss were determined by change of conductivity. This measure is open to the objection that no account is taken of other ions which may pass into the solutions from the disk. Analysis of the solutions for potassium and chloride showed that this is not a serious complication, particularly after the first few hours. Accordingly, the results for the early stages are not used. There is the same uncertainty about the effective area of the disk as in the diffusion experiments with carbon dioxide. The following are the results of a typical experiment which lasted for 90 hr. The disk was first exposed to distilled water and after some hours, when the rate of change of conductivity became negligible, potassium chloride was added in sufficient quantity to bring the concentration on one side up to 0·1M. At the end of the experiment this had fallen to 0·097M, while the conductivity of the weaker solution had risen by an amount equivalent to an increase of about 0·005M-KCl. Both the stronger and weaker solutions, which were equal in volume, increased in conductivity during the first period of 17½ hr. Neglecting this period, the values of $K \times 10^7$ for successive periods were 3·6, 3·1, 3·1, 3·6 and 3·6 cm.^{2} sec.$^{-1}$, and those for $h \times 10^5$ were 2·5, 1·5, 1·4, 1·3 and 0·55 sec.$^{-1}$ The variation in the values is partly due to experimental error in determining the uptake which is a small difference between two measurements. The values for the whole period are $K = 3{\cdot}2 \times 10^{-7}$ and $h = 1{\cdot}2 \times 10^{-5}$. Replicate experiments gave mean values of 2·3 and $4{\cdot}15 \times 10^{-7}$ for K, and 2·86 and $1{\cdot}72 \times 10^{-5}$ for h. The calculated values for the coefficient of diffusion differ by less than 10% if the absorption is assumed to be independent of concentration.

In another set of experiments the disks were placed in tap water which had been boiled and, after the rate of change of conductivity had reached a low value, potassium chloride was added to one side in sufficient quantity to bring the concentration up to 0·01M. This experiment was carried out in duplicate. The average values for K from 17 to 70 hr. were the same for both, $2{\cdot}0 \times 10^{-7}$, those for h, 1·65 and $0{\cdot}395 \times 10^{-5}$. The variability of the results is such that we cannot say that there is any significant change of the constants with change of concentration.

The coefficient of diffusion of potassium chloride in carrot tissue is about one-fiftieth of that in water, thus indicating that the path in the tissue, which consists largely of water, is considerably restricted.* Measurements of the potential difference between 0·1 M and 0·01 M-KCl, separated by a disk of carrot tissue, showed that the weaker side was 43·5 mV. positive. This is the type of result we should expect if part of the medium through which the KCl passed consisted of an electrolyte with non-mobile anions. There is evidence that the cytoplasm is such (Briggs, 1932). The effect of such a phase on the potential difference and the coefficient of diffusion in the tissue will be discussed in Appendix B.

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* For a homogeneous disk placed in a solution, the time required for the concentration in the median plane to rise half-way from its original value to its final value is inversely proportional to K for the disk. For tissue where the solute moves freely in one phase then, it is the value of K in that phase and not the value for the tissue as a whole which determines the time for the rise of concentration in that phase. Absorption from that phase will shorten the time. For disks of 0·1 cm. thickness the time is of the order of 5 min. for KCl if the coefficient of diffusion, in the phase where it moves readily, is the same as in water.

It will be seen that, in general, the coefficient of diffusion of an electrolyte in a membrane containing non-mobile anions is greater than the apparent coefficient, measured in terms of the electrolyte outside the membrane—how much greater depends upon the concentration of the electrolyte relative to that of the non-mobile anion and upon the ionic mobilities. This makes the problem much more complicated. In the absence of the necessary data for a full treatment the complications have been neglected.

The experiments of Stiles & Skelding (1940*b*) suggest that in the early stages of the absorption of manganese chloride by carrot tissue much of the absorption of the cation, originally absent from the tissue, is in exchange for cations already present in the tissue. For such an exchange of cations the movement of the ions in the tissue may be much easier than it is when the absorption of cation is accompanied by a more slowly moving anion, as in the absorption of potassium chloride. A 'diffusion' experiment with methylene blue hydrochloride gave some results which, although not very reliable, are of interest in this connexion. Other evidence suggests that in the early stages much of the uptake of the cation of methylene blue is in exchange for some cation of greater mobility. The methylene blue was determined colorimetrically and the results give a value of $2{\cdot}7 \times 10^{-6}$ cm.^{2} sec.$^{-1}$ for the diffusion constant, nearly ten times as great as that for potassium chloride.

(2) '*Absorption*' *experiments*

The above 'diffusion' experiments were carried out at 12° C., while the 'absorption' experiments now to be considered were carried out at a temperature 10° higher. In one of these with $c_0 = 0{\cdot}0095$ M-KCl at $t=0$ falling to 0·0046 M after 80 hr., the calculated value of $h \times 10^5$ fell from 10·5 in the first 10 hr. to 4·9 in the next 10 hr. and eventually to 2·7. The assumed value for K, used in arriving at h, was $3{\cdot}8 \times 10^{-7}$ instead of 3·1, the average value for 12° C. This is based on the assumption that the coefficient of diffusion in the tissue is increased by temperature as much as it is in water. Experiments to decide whether this assumption is justified were unsuccessful. The results were very variable and there was loss of electrolyte from the tissue at the higher temperatures. Further, our estimates of h are necessarily approximate since the value of K varies with the sample of tissue. It may be noted that if we had used 3·1 instead of 3·8 in the value for K the values of h would not have been more than 10% higher.

In any comparison of the values of h derived from the 'diffusion' and 'absorption' experiments it must be remembered that those from the former are too big by about 30% on account of the disk area exceeding the corrected area used in the calculation of the coefficient of diffusion. Further, the 'absorption' experiments show that with the higher concentrations there is a fall of h with time, a fall which is difficult to detect in the 'diffusion' experiments on account of the rather large experimental error.

If the rate of absorption by cells increases nearly proportionally with increase of concentration when the solution is dilute, and less rapidly for higher concentrations, until eventually a concentration is reached where the rate of absorption attains a maximum, we should expect h (a ratio of rate to concentration) to approach a maximum for dilute solutions and to fall as the concentration is increased. Indications of a higher value for h

with lower concentrations is provided by the fact that eleven experiments with 0·1 cm. disks in 0·01 M-KCl showed a mean absorption of 2·1 units in the first 5 hr. with a standard deviation of the mean of 0·06, while six experiments with 0·005 M showed a mean of 1·46 with a standard deviation of 0·19. If *h* were independent of *c* then the second rate should be about half the first, i.e. 1. If concentration were the only factor determining *h*, we should expect *h* to rise as the external concentration is reduced by absorption. The cells cannot, however, continue absorbing indefinitely, and eventually the rate of absorption falls to zero while there is still a finite concentration outside. Whatever may be the explanation of this—the running down of an absorption mechanism or increasing leakage as an internal concentration is built up—this factor will tend to cause a fall of *h* with time. The fall of *h* with time in the experiments with 0·01 M, despite the fall of concentration, may indicate the dominance of this second factor. In the experiments with initial concentrations lower than 0·01 M, the value of *h* rises during the course of the experiment. With 0·005 M it rose from 17·4 to $22{\cdot}6 \times 10^{-5}$ sec.$^{-1}$ in the first 20 hr., while in an experiment with 0·002 M the rise was greater, from 27 to 65×10^{-5} sec.$^{-1}$ in 30 hr.* We might expect such a difference in the drift of *h*, since in a given period of time the smaller the concentration the less is the amount absorbed and the greater is the percentage fall in the external concentration.

In the above calculations we have assumed a value for the coefficient of diffusion, based on the results of the 'diffusion' experiments, in order that we might arrive at an estimate of *h*. This would not have been necessary if we had data for the absorption by disks so thin that the difference between the concentration at the centre and the surface of the disk was negligible. For such disks *h* is the rate of absorption per cm.^{3} of disk divided by the concentration. This direct estimate of *h* can then be used to derive the value of *K* from the results with thicker disks. The results of such a calculation are shown in the following:

In one experiment disks of thickness 0·044, 0·094 and 0·194 cm. (after allowing for the cut surface cells) were placed in 0·01 M-KCl. The material was as nearly comparable as possible, since thin, medium and thick disks were cut in succession repeated forty times to get forty disks of each thickness. Assuming that the thinnest disks are suitable for such a direct calculation of *h* the following values were obtained: $7{\cdot}9 \times 10^{-5}$ sec.$^{-1}$ in the first 10 hr. falling to $3{\cdot}3 \times 10^{-5}$ in the fourth. For such values of *h* it can be calculated (see p. 275) that the difference between the concentration at the centre of the disk and at the surface is less than 6%. The next step is to ascertain what value of *K*, in conjunction with this value of *h*, will give us a calculated value for the absorption for the thicker disks equal to that observed. But here we are faced with the complication that *h* is greater the smaller the concentration and that the concentration at the centre of the thicker disks is appreciably less than at the surface. Taking *h* as $7{\cdot}9 \times 10^{-5}$ sec.$^{-1}$ and trying 4×10^{-7} cm.^{2} sec.$^{-1}$ as a value for *K*, we calculate a rate of 4·95 units in the first 10 hr. by the 0·194 cm. disks as compared with the observed rate of absorption of 5·5 units. The value of *h* has to be increased to $9{\cdot}5 \times 10^{-5}$ to make the calculated and observed rates agree and still leave the value for the coefficient of diffusion of the order of that suggested by the diffusion experiments. While there are indications that *h* ought to be taken at a higher value for the thicker disks, we cannot say how much higher. We may conclude, however, that the results of the absorption experiments are not inconsistent with those of the diffusion experiments when allowance has been made for the difference of the conditions.

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* In the former experiment the external concentration fell to 0·0032 and in the latter to 0·0006.

Some of the experiments by Stiles & Skelding (1940*a*) on the absorption of potassium chloride by 0·1 cm. disks of carrot were done under conditions very similar to those of our 'absorption' experiments, except for the shorter washing period. Since they measured the absorption of the two ions directly, and not the change of conductivity as we did, it is of interest to compare their results with ours. According to their Table III, 13 % of the potassium and 11 % of the chloride were absorbed from 0·02 M-KCl in the first 18·9 hr. Assuming $K = 3{\cdot}8 \times 10^{-7}$ cm.^{2} sec.$^{-1}$ this gives a value for h of $2{\cdot}1 \times 10^{-5}$ while in our experiment with a solution half as strong the value was $10{\cdot}5 \times 10^{-5}$ during the first 10 hr., falling to $6{\cdot}9 \times 10^{-5}$ in the next ten. Their experiment with 0·005 M gives a value for h of $9{\cdot}5 \times 10^{-5}$ as compared with $17{\cdot}4 \times 10^{-5}$ in the first 5 hr. rising to $22{\cdot}6 \times 10^{-5}$ in the second 10 hr. from our experiment at the same concentration. The smaller value of h in the experiments of Stiles & Skelding may be partly due to difference in material, but we should expect a difference of this kind on account of the difference in pre-treatment (see p. 278).

Concentration of salt in the disk

In considering the fall of concentration of the solute being absorbed with distance from the surface of the disk we shall take as an index the difference between the concentration at the central plane, $c_{\frac{1}{2}}$, and that at the surface, c_0. We must keep in mind that, since h rises as c falls, $c_{\frac{1}{2}}$ will be lower than our value calculated on the assumption that h is independent of x, and also note that the gradient of concentration is steepest at the surface falling to zero at the centre. Putting $x = l/2$ in equation (9) we get

$$c_{\frac{1}{2}} = c_0 \frac{\sinh\frac{\mu l}{2} + \sinh\frac{\mu l}{2}}{\sinh \mu l} = \frac{c_0}{\cosh\frac{\mu l}{2}}.$$

In the absorption experiment with 0·0095 M-KCl, h was $10{\cdot}5 \times 10^{-5}$ sec.$^{-1}$ at the beginning. This being equivalent to $\mu = 16{\cdot}5$, the value of $c_{\frac{1}{2}}$ for a disk of 0·1 cm. is 73·5 % of the surface concentration, but as h falls with time the concentration at the centre rises until it is 91·6 %. The percentage difference in concentration between centre and surface not only is greater for more dilute solutions but also increases with time; for 0·002 M-KCl $c_{\frac{1}{2}}$ is 49 % of c_0 at the beginning and falls to 3 % after 30 hr. The difference would be greater for thicker disks.

In the light of the above considerations it is not surprising that in some absorption experiments with disks of different thickness, the rate of absorption per disk fails to maintain proportionality to the bulk of the disk. Commenting on such a failure of potato disks in KBr solutions Steward, Wright & Berry (1932) suggest that the explanation is that absorption is limited to the cells in the surface layer, 'but', they say, 'it must not be supposed that the further penetration of bromide is limited by its inability to diffuse through the tissue, for in another investigation Steward has shown that it may pass in appreciable quantity *through* a mounted membrane 1·5 mm. thick in periods much shorter than used in these experiments'. The best way to approach the problem is to use the available information about the size of constants of diffusion and absorption to estimate the difference between the concentration within and outside the disk, always keeping in mind the simplification of the assumptions necessary for the solution of the equations. The diffusion experiments referred to above are those of Steward (1930) with potato and potassium phosphate in the presence of various concentrations of glucose. With the most dilute sugar (0·044 M) present the amount of phosphate coming out of the disk was more than twice as great as that entering. The determination of the latter quantity is subject to greater experimental error than the former. Steward neglects any absorption or production by the disk and calculates the coefficient of diffusion of phosphate as $2·2 \times 10^{-9}$ cm.^{2} sec.$^{-1}$ Allowance for the area of the disk covered by the flange, as for our diffusion experiments, would reduce the 2·2 to about 1·6. The experiment with 0·111 M glucose present shows 11 units of phosphate as entering and only 0·269 as passing through the disk. Steward, again neglecting the retention of 10·7, calculates K as $5·58 \times 10^{-9}$; allowance for the disk under the flange reduces the 5·58 to 4·1. If the retention is taken into account, assuming h constant, the 4·1 is then raised to 37.* In view of the fact that in none of the other experiments was the retention as high as 10·7 units the figure of 37×10^{-9} cm.^{2} sec.$^{-1}$ is probably a considerable overestimate of the value of K for phosphate in the tissue in the absence of sugar. The presence of sugar, probably by decreasing the volume of the cells and increasing the volume of the liquid in the intercellular spaces, causes an increase in the value of K for the tissue. Granting their assumption that the coefficient of diffusion of phosphate may be used as a measure of that of bromide, we can estimate the extent to which the uptake of bromide is limited by its ability to diffuse in the tissue. Using their data in Table 12 (Steward *et al.* 1932), we calculate an absorption of $2·75 \times 10^{-12}$ equiv./cm.^{2}/sec. for potato disks of 1·14 mm. thickness, taking the area of the disk as that of the plane surfaces. Assuming h is constant this gives a value for μ of 100 cm.$^{-1}$ and a concentration at the centre of the disk less than 1 % of that at the surface. It may well be smaller, since we have taken our highest estimate of K (a figure sixteen times Steward's estimate) and neglected any rise of h towards the centre of the disk consequent upon fall of c. The observation that disks of 0·33 and 1·58 mm. absorb at practically the same rate per disk as those of 1·14 is not unexpected since, using the values of the constants as above, the ratio of the rates of absorption should be as 0·925 to 1·00 to 1·00. The experiments with yet thicker disks show an increase in the rate with thickness. Their Table 13 shows that a disk of 9·85 mm. absorbed at 1·47 times the rate of one of 1·7 mm., but this is probably to be explained by the fact that while, with thin disks, the curved edge can be neglected in considering the surface of the disk, this ceases to be justified for thick disks. It is striking that the ratio of the *total* surface of the 9·85 and 1·7 mm. disks is very nearly the same as the ratio of the rates of absorption; it is 1·49.

We are not maintaining that their assumption of a difference in the absorptive capacity of the surface and inner cells is wrong, but merely that it is unnecessary in view of the possible dimensions of the coefficient of diffusion and the consequent marked fall of concentration as the centre is approached; it is to the results of the diffusion experiments which they appeal for support. Such a difference in absorption capacity, at high concentrations at least, could be demonstrated if the difference between thin and thick disks in rate of absorption per bulk persisted at external concentrations so high that even the concentration at the centre of the disk was in the region where absorption is almost independent of concentration. This might prove impossible with the thickest discs because the high concentrations required might depress the absorption by the outer cells.

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* This value for K is only about one-tenth of the value of K for KCl in carrot tissue.

Steward *et al.* appeal also to the general parallelism between absorption and respiration. They have shown that there is some evidence for a difference in the respiratory activity of surface and inner cells and, as we have seen, this is not likely to be due to shortage of oxygen for the latter.* Any parallelism between respiratory and absorptive capacity of cells as distance from the surface of the disk increases, which may exist in potato tissue, does not extend to carrot tissue such as used by us. The experiments with disks of different thicknesses referred to on p. 275 showed respiration per disk in the proportion of 0·43 to 1·00 to 1·98, while the proportion of thickness of living tissue was 0·47 to 1·00 to 2·07, thus suggesting that the cells at the centre of even a 2 mm. disk respire at the same rate as those at the surface. When we turn to the absorption of potassium chloride by the same disks from a 0·01 M solution, we find that the rate per disk fails to keep up with the increase of thickness; the proportion of the rates of absorption is 0·48 to 1·00 to 1·52. As we have seen this differs only little from that calculated on the assumption that the absorption by the cells, whatever their distance from the surface of the disk, is proportional to the concentration outside the cell. The marked difference between potato and carrot disks as regards the relation between the rate of absorption and disk thickness can be attributed mainly to the difference in the resistance offered by the tissue to diffusion of the solute. The value for h for the potato disks in 0·00075 M-KBr is 37×10^{-5} cm.$^{-1}$ while that for carrot disks in 0·00075 M-KCl is about 60×10^{-5}. The results of Stiles & Skelding (1940*a*) suggest that carrot disks absorb potassium bromide at about the same rate as they do potassium chloride.

In view of the uncertainty as to the relation between h and concentration, the difficulty of solving complicated differential equations, and the difficulty of cutting very thin disks, we must conclude that deductions from the results of experiments with carrot disks are on a more secure foundation than are those from results of experiments with potato disks where the concentration falls off so much more rapidly as the centre is approached.

PREPARATION OF MATERIAL AND TREATMENT IN ABSORPTION EXPERIMENTS

Since our purpose is to deduce the attributes of the cells and the state of their environment from the results of experiments with disks of tissue, we need not make special claims about the conditions of our absorption experiments and we have deferred until this stage any reference to experimental procedure. The disks were arranged in stainless steel wire cages so that practically the whole surface of the disks was exposed to the solution which was aerated with a stream of air freed from carbon dioxide. Increase of the speed of the air current did not increase the rate of absorption. Under these conditions the tissue could be maintained fresh and capable of absorption for several weeks. Stiles & Skelding (1940*a*) have argued that the conditions in their experiments were as favourable for absorption as those used by Steward and his collaborators and it may be noted that, although the temperature for our experiments was only one degree higher, the rate of absorption per cm.^{2} of disk surface was about twice as great. This difference may be due partly to the nature of the carrot material as well as to the difference in pretreatment.

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* There is considerable evidence for the view that rate of respiration rises with rate of salt absorption. If concentration of salt outside a cell is the only factor determining the rate of absorption by the cell then, as we have seen, Steward's results might be explained by the fall of concentration within the disk, and this would entail a fall in respiration without any limitation by oxygen supply (see p. 270). Whatever may be the full explanation of the results the above points should be taken into account.

Any comparison of our results with those from absorption experiments which appear to be similar should take into account the following points. The procedure adopted by Steward and by Stiles is to give the material a preliminary washing in tap water for about 24 hr. with a brief washing in distilled water. Steward & Harrison (1939) state that under such conditions potato disks continue to absorb from a dilute solution at a steady rate for many hours. Stiles's results with carrot tissue indicate that 'the rate of absorption, rapid at first, gradually declines. This course does not, however, continue, and gives place to a phase during which the rate of absorption increases.' Experiments in this laboratory under similar conditions show the same changing rate. Simultaneous measurements of the rate of respiration showed that the phase of increasing rate of absorption was one of decreasing rate of respiration. If, after a brief washing in tap water, the carrot disks are kept in aerated distilled water until such time as the respiration, at first high, falls to a more or less steady value, then the rate of absorption is initially higher than when the respiration is high and shows an uninterrupted fall as the concentration outside falls and that in the tissue rises (Robertson, 1941). Disks which had been 9 days in distilled water showed a fall in rate of absorption which was indistinguishable from that with disks which had been in distilled water for 6 days. The results recorded here are from tissues which had attained this steady rate of respiration while being kept in aerated distilled water. Hence, the difference, mentioned on p. 275, between our data and those recorded by Stiles & Skelding may well be due to the difference in the pretreatment of the tissue. We do not propose to discuss now the possible explanations of the above differences between potato and carrot disks, but we may recall that while h is not markedly different for the two tissues K is about ten times greater for carrot tissue.

CONCLUSION

In conclusion we may review the progress we have made in the solution of the problem of gaining information about the activity of cells from results of experiments on the activity of aggregates of the cells. Having formulated the problems we have considered the question of absorption by disks of tissue and can conclude as follows: When the absorption by the cells, p, is independent of the concentration around them, c, the absorption by the disk is also p and is independent of the concentration at the surface, c_0, whatever the thickness of the disk—by absorption is meant the rate per cm.^{3} Judging from rates of production of carbon dioxide, this probably applies to the absorption of oxygen by disks of carrot up to 0·2 cm. in thickness. The values of K, the coefficient of diffusion, and p can be obtained from 'diffusion' experiments where the concentrations on the two sides of the disk are different, and p from 'absorption' experiments where the concentrations are the same. The concentration inside the disk at different distances from the surface can be calculated. Although this is not significant for the absorption of the substance, it may be of importance if the concentration affects the rates of other metabolic processes.

When absorption by the cells is proportional to c, that is $p=hc$, the absorption by the disk is less than pc_0 but is proportional to it. The proportionality factor falls as h relative to K, and l, the thickness of the disk, increase. The values of K and h can be deduced from 'diffusion' experiments and, knowing K, h can be calculated from 'absorption' experiments. Hence values of c for different distances from the surface can be calculated. Experimental data on diffusion and absorption of salts have been considered from this point of view. Here also, there is the effect of the concentration of a substance not only on the absorption of the substance, but on other processes, direct and indirect. The effect of concentration of salt on the absorption of salt and the interrelation of salt absorption and respiration is an example which is considered. Absorption of oxygen could be treated in the same way. If p is a linear function of c, say $hc+g$, it can be shown that the absorption by the disk is a linear function of c_0, mhc_0+mg, where m is a fraction. The concentration for a given rate of absorption per cm.^{3} of disk is no longer a constant multiple of the concentration for the same rate for the cell. Hence it follows that when the equation for the absorption-concentration relation for the cell has a curved form, the equation for the relation for the disk can no longer be obtained by substituting mc_0 for c. The simplest way to determine the former relation will be to use disks which are so thin that the concentration for the central cells is not appreciably different from that for the surface cells. The alternative of deducing the relation for cells from the relation for disks by assuming the form and then determining its parameters is simple when the relation is one of proportionality, but complex for other relations.

In addition, we have considered the problem where the absorption, p, while independent of c, falls off linearly with distance from the surface. Other variations could be considered but the analysis might become very lengthy.

The values of diffusion and absorption constants and of concentrations within the disks depend upon the validity of the assumptions. For the experimental data discussed, the assumptions, made for mathematical simplicity, only approximate to the truth and the deductions are accordingly approximate.

APPENDIX A

Effective area of disk in diffusion experiments

The disk was secured between the two vessels, A and B, by clamped flanges, and the edges sealed with luting wax, S (see Fig. 1). In the experiments with KCl the chambers were partially filled and the whole was rotated to ensure efficient aeration of the disk. In the experiments with CO_2 the chambers were filled and a stream of air containing CO_2 was passed through one while CO_2-free air was passed through the other.

An *approximate* value for the effective area of the disk is obtained as follows: for all that part of the disk not covered by the flange the effective length of the diffusion path is assumed equal to the thickness of the disk. For an annular disk of thickness df (measured in the plane of the disk) and distance f within the flange the effective path is assumed to be abc, i.e. $\sqrt{(4f^2+l^2)}$. The amount diffusing through that part of the disk not covered by the flange will be proportional to $\pi r^2/l$ and that through the part covered by the flange $\int_0^F \frac{2\pi(r+f)\,df}{\sqrt{(4f^2+l^2)}}$. The latter multiplied by l will give the amount to be added to πr^2 to give the effective area. In our experiments this amounted to about 30% of πr^2, while the total area under the flange was about 60%.

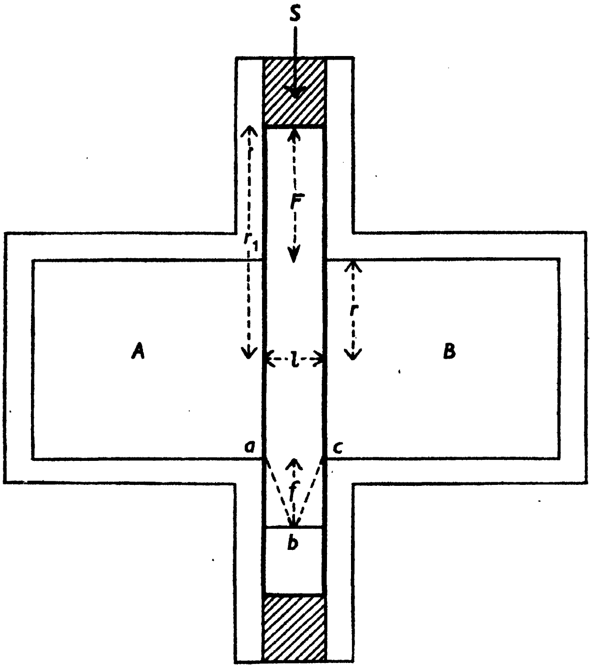

Fig. 1.

APPENDIX B

Diffusion of an electrolyte through a membrane containing non-mobile anions

For the sake of simplicity activities will be taken as equal to concentrations.

Let

A = the activity of non-mobile anions in the membrane.

c_1 and c_2 = the activities of univalent anion and cation of an electrolyte on the two sides of the membrane.

p = the activity of cation within the membrane; p_1 and p_2 at the two surfaces of the membrane.

$p-A$ = the activity of the anion.

dE/dx = the potential gradient within the membrane.

u = the mobility of the cation within the membrane.

v = the mobility of the anion.

The rate of movement of cation, ρ, will be given by

$$\rho = \mu p\left[-\frac{RT}{p}\frac{dp}{dx} - F\frac{dE}{dx}\right],$$

and that of the anion, which must be the same as that of the cation, by

$$\rho = v(p-A)\left[-\frac{RT}{p-A}\frac{dp}{dx} + F\frac{dE}{dx}\right].$$

From these expressions we can find the potential gradient and by integration the difference of potential, $E_1 - E_2$, between the two surfaces of the membrane.

$$\frac{dE}{dx} = -\frac{RT}{F}\frac{dp}{dx}\frac{u-v}{(u+v)p-vA},$$

$$E_1 - E_2 = \frac{u-v}{u+v}\frac{RT}{F}\log_e\frac{(u+v)p_2-vA}{(u+v)p_1-vA}.$$

The potential differences between solution and membrane are given by

$$\frac{RT}{F}\log_e\frac{A+\sqrt{(A^2+4C_1^2)}}{2C_1} \quad \text{and} \quad \frac{RT}{F}\log_e\frac{2C_2}{A+\sqrt{(A^2+4C_2^2)}}.$$

Therefore the total difference of potential between the two solutions is

$$\frac{RT}{F}\left[\log_e\frac{C_2}{C_1}\frac{A+\sqrt{(A^2+4C_1^2)}}{A+\sqrt{(A^2+4C_2^2)}} + \frac{u-v}{u+v}\log_e\frac{(u+v)p_2-vA}{(u+v)p_1-vA}\right].$$

This is the expression given by Teorell (1935) in a paper on the theory of membrane permeability, which is restricted to the consideration of the topic of potential differences.

The expression for the rate of movement of electrolyte through the membrane can be obtained by substituting in the equation for ρ the value for dE/dx and we obtain

$$\rho = -uRT\frac{dp}{dx}\left[\frac{2vp-vA}{(u+v)p-vA}\right].$$

Hence the coefficient of diffusion within the membrane is $uRT\frac{2vp-vA}{(u+v)p-vA}$, as compared with $\frac{2uv}{u+v}RT$, the value in water. The former depends on the values of p and A and hence upon x, except when $u=v$. The mobilities of the ions may be different in the membrane from what they are in water. The value of the coefficient in the membrane depends upon the values of A[*] and p except in the case when the mobilities of anion and cation are equal.

In the experiments discussed in this paper it is not the coefficient of diffusion in the membrane which is calculated but $\rho\frac{x_2-x_1}{c_2-c_1}$,[†] which may be called the apparent coefficient of diffusion in the membrane. The expression for the apparent coefficient is obtained by first integrating the differential equation for rate of movement of electrolyte through the membrane

$$\rho\frac{x_2-x_1}{p_2-p_1} = \frac{2uv}{u+v}RT\left[1 - \frac{u-v}{u+v}\frac{A}{2(p_2-p_1)}\log_e\frac{p_2-\frac{vA}{u+v}}{p_1-\frac{vA}{u+v}}\right]$$

and then substituting for p values of c calculated from

$$c^2 = p(p-A).$$

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[*] The experiments of Stiles & Adair (1921) on diffusion of NaCl in gelatin of different strengths are not likely to show any marked effect of the concentration of A on the diffusion, since the concentration of A in 10% gelatin is probably of the order of 10^{-3} which is very small compared with the concentration of the diffusing electrolyte.

[†] For the purposes of simplification it will be convenient to neglect absorption by the cells.

If, for an electrolyte such as KCl, the mobilities of the two ions in the membrane are equal, as they are in water, then the coefficient of diffusion within the membrane is uRT. This is the same as the value of the coefficient in water if the mobilities are the same in the membrane as they are in water. While the value of the coefficient is given by $\rho\frac{x_2-x_1}{p_2-p_1}$, that of the apparent coefficient is a fraction $(p_2-p_1)/(c_2-c_1)$ of the real coefficient. The following table shows how the value of the apparent coefficient falls with decrease of the ratio c/A:

A	c_2	c_1	$\frac{p_2-p_1}{c_2-c_1}$
0·1	0·10	0·010	0·680
0·1	0·01	0·001	0·110
0·1	0·10	0·001	0·620
1·0	0·10	0·010	0·110
1·0	0·01	0·001	0·011

For electrolytes with ions of unequal mobilities the coefficient of diffusion in water is $\frac{2uv}{u+v}RT$, and if the mobilities in the membrane are the same as in water the value of $\rho\frac{x_2-x_1}{p_2-p_1}$ is less or greater than the value of the coefficient in water according as v is less or greater than u. The ratio p/c is not dependent upon mobilities. The mobility of H_2PO_4 ions in water is 0·77 of that of K ions. Hence, assuming mobilities in the membrane the same as in water, the apparent coefficient of diffusion of KH_2PO_4 with A at 0·1, c_2 at 0·1 and c_1 at 0·01 is 63% of the coefficient of diffusion in water while that for KCl is 68%.

If a slice of carrot tissue behaved like a membrane, such as we have considered above, then we could, assuming $u=v$, deduce a value of about 0·1 N for A from the fact that a 0·01 M solution of KCl on one side is 43·5 mV. positive to a 0·1 M solution on the other. If the mobilities were the same in the tissue as in water we could then proceed to calculate the apparent coefficient of diffusion of KCl in the tissue, which would depend upon the concentration of salt in the solution on the two sides in the way we have indicated. The carrot slice is more complicated than our simple membrane. The general evidence suggests that movement of ions is restricted to the cytoplasm and the wet walls and intercellular spaces as far as these are filled with water. The restriction to part of the disk will reduce the value of the apparent coefficient of diffusion in the disk and, in as far as the cytoplasm behaves like our simple membrane, the addition of a water-path without non-mobile ions will result in the potential difference across the disk being intermediate between that for a membrane without non-mobile ions and that for a membrane with such ions. Hence our observed potential difference of 43·5 mV. would suggest a concentration of non-mobile anions in the cytoplasm greater than 0·1 N.

Although the expression for the potential difference across a membrane consisting partly of a phase with non-mobile ions and partly of water can readily be obtained, it is much too complicated to be of use in determining the concentration of non-mobile ions from the observed potential difference across the membrane.

There are other ways in which the ideal system we have discussed differs from what may be realized in practice. We, like Teorell (1935), have considered a membrane where the concentration of non-mobile ions is the same throughout the membrane. If these ions are produced from an acid then their concentration will not be the same throughout. Since the ratio of mobile cations in the membrane surface to that in the solution adjoining, p/c, is greater for the side where c is smaller then, unless suitable adjustments are made in the concentration of H ions in the external solution, the concentration of H ions will be greater, and of non-mobile anions smaller, at this surface than at the other where the external solution is stronger.

The equations for gradient of potential and for rate of movement of ions in terms of u, v, and gradients of p and A can readily be formulated. To obtain the total potential difference across the membrane and the rate of movement of ions in terms of the concentrations at the two sides is complicated, and involves a knowledge of the dissociation constant of the acid producing the non-mobile anions.

REFERENCES

Briggs, G. E. (1932). The absorption of salts by plant tissues considered as ionic interchange. *Ann. Bot., Lond.*, **46**, 301–22.

Robertson, R. N. (1941). Studies in the metabolism of plant cells. I. Accumulation of chlorides by plant cells and its relation to respiration. *Aust. J. Exp. Biol. Med. Sci.* **19**, 265–78.

Steward, F. C. (1930). Diffusion of certain solutes through membranes of living plant cells and its bearing upon certain problems of solute movement in the plant. *Protoplasma*, **11**, 521–57.

Steward, F. C. (1932). The absorption and accumulation of solutes by living plant cells. IV. Surface effects with storage tissue. A quantitative interpretation with respect to respiration and salt absorption. *Protoplasma*, **17**, 436–53.

Steward, F. C. & Harrison, J. A. (1939). The absorption and accumulation of salts by living plant cells. IX. The absorption of rubidium bromide by potato discs. *Ann. Bot., Lond.*, N.S. **3**, 427–53.

Steward, F. C., Wright, R. & Berry, W. E. (1932). The absorption and accumulation of solutes by living plant cells. III. The respiration of cut discs of potato tuber in air and immersed in water, with observations on surface:volume effects and salt accumulation. *Protoplasma*, **16**, 576–611.

Stiles, W. & Adair, G. S. (1921). The penetration of electrolytes into gels. III. The influence of the concentration of the gel on the diffusion of sodium chloride. *Biochem. J.* **15**, 620.

Stiles, W. & Skelding, A. D. (1940*a*). The salt relations of plant tissues. I. The absorption of potassium salts by storage tissue. *Ann. Bot., Lond.*, N.S. **4**, 329–63.

Stiles, W. & Skelding, A. D. (1940*b*). The salt relations of plant tissues. II. The absorption of manganese salts by storage tissue. *Ann. Bot., Lond.*, N.S. **4**, 673–700.

Teorell, T. (1935). An attempt to formulate a quantitative theory of membrane permeability. *Proc. Soc. Exp. Biol., N.Y.*, **33**, 282–5.

THE EFFECT ON STOMATAL BEHAVIOUR OF DETACHING LEAVES OF *KALANCHOË* AND *MESEMBRYANTHEMUM* AT DIFFERENT TIMES OF THE DAY

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(With 2 figures in the text)

INTRODUCTION

Detached leaf culture has for a long time been a convenient technique for many plant physiological, phytopathological and entomological studies. Although the physiology of detached leaves and the effect of detachment on the normal life processes have been fairly well studied, yet very little work has been done to elucidate the influence of detachment on the stomatal movements. Darwin (1911, 1916) and Laidlaw & Knight (1916) observed a temporary rise in the transpiration rate of detached leaves, and interpreted this rise as due to a sudden increase in the opening of the stomata caused by release of water tension on detaching the leaves. Such increased opening of stomata was, however, not confirmed by the direct observations of Loftfield (1921), nor by indirect determinations of Krause (1935).

Oppenheimer & Mendel (1939), using Pinkhof's (1920) porometer, found no appreciable change in the porometer times during the first 10 min. after the leaves had been detached. After 10 min., however, the porometer times were found to increase markedly, indicating a reduction in the stomatal apertures until the latter became almost completely closed after 1 hr. from severing the leaves. These authors did not follow the stomatal movements for more than 1 hr. after leaf detachment.

The present investigation is an endeavour to compare, for prolonged periods, the width of stomatal apertures of detached leaves with that of leaves still attached to the plant. The plants used were *Kalanchoë marmorata* Baker. and *Mesembryanthemum forskalei* Hochst.

EXPERIMENTATION AND RESULTS

Determination of the stomatal apertures

Lloyd's alcohol fixation method (1908) was used; strips were taken only from the underside of the young leaves, usually from just behind the tip, a region which was observed by Desai (1937) to have particularly sensitive stomata. For each determination three strips were taken from three different leaves of about the same age, ten stomata were chosen at random from each strip, and their widths were determined by means of a standardized ocular micrometer. Only the mean values of the thirty measurements are recorded in the figures.

Stomatal behaviour of attached and severed leaves in relation to the time of severing

Severing of the leaves was done at times when the stomata were normally approaching their maximal and minimal widths. Thus, in the case of *Kalanchoë marmorata* Baker., the leaves were severed at 7.15 a.m., 11.45 a.m., 8.00 p.m., and 12 m.n.; in *Mesembryanthemum forskalei* Hochst., on the other hand, the leaves were severed at 8.30 a.m., 2.15 p.m., and 11.00 p.m. Immediately after plucking the leaves their cut ends were

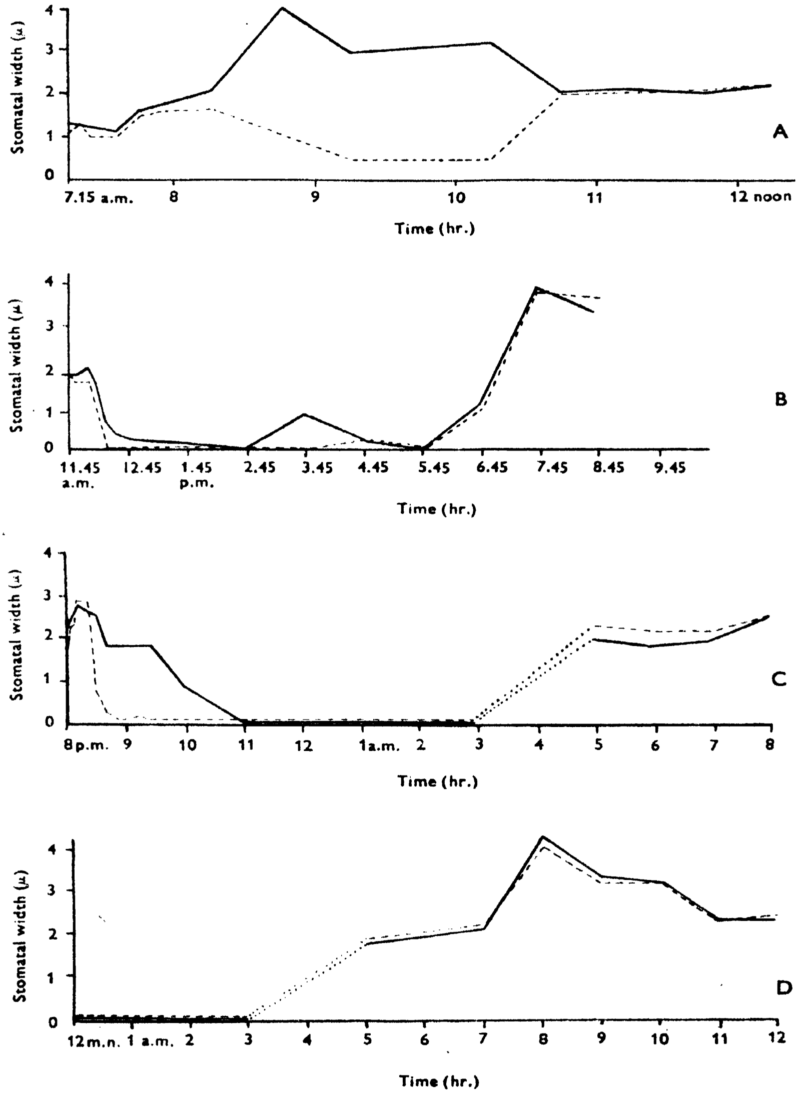

Fig. 1. Stomatal behaviour of attached leaves of *Kalanchoë* as compared to that of leaves detached at (A) 7.15 a.m., (B) 11.45 a.m., (C) 8.00 p.m. and (D) 12 m.n. Continuous line for attached leaves and dotted line for detached ones. No measurements were done between 3 and 5 a.m.

immersed in water, and the stomatal movements were followed first every 5 min. and then the time of observation was gradually increased until it became hourly. In the meantime, stomatal behaviour of the still attached leaves was also studied at the specified time intervals. These observations continued for different lengths of time which varied from 5 to 12 hr. The average values of the stomatal width of both attached and detached leaves are represented graphically in Figs. 1 and 2 for *Kalanchoë* and *Mesembryanthemum* respectively.

A glimpse at the figures reveals that if the leaves were severed at a time when their stomata were approaching their maximal aperture, severing always induced closing of the stomata, but the closing lagged a little behind the time of leaf detachment (Figs. 1 A, C and 2 A, B). The effect of severing usually lasted for a few hours (between 2–7 hr.), after which the stomata of both attached and detached leaves followed the same rhythmic course. On the other hand, if severing took place at times when the stomata were tending to close, or were completely closed, then severing had little or no effect on the stomatal movements (Fig. 1 B, D).

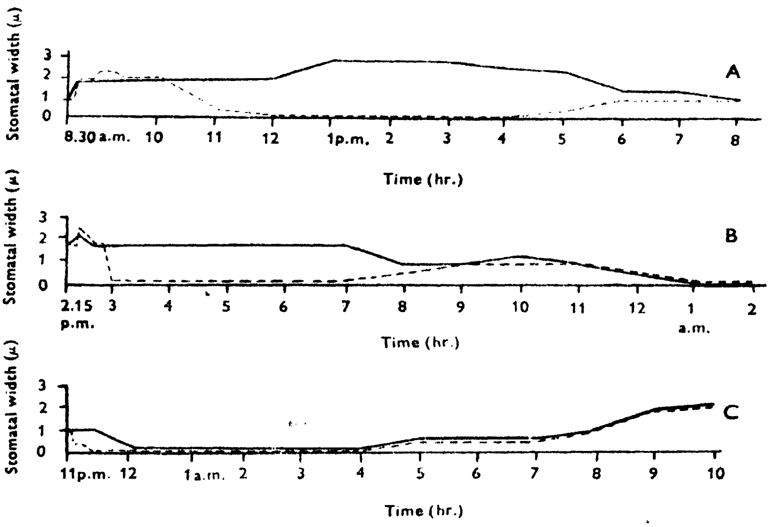

Fig. 2. Stomatal behaviour of attached leaves of *Mesembryanthemum* as compared to that of leaves detached at (A) 8.30 a.m., (B) 2.15 p.m. and (C) 11 p.m. Continuous line for attached leaves and dotted line for detached ones.

In all probability the closing of stomata as a result of leaf detachment is a sort of 'Shock response'. The shock progressively decreases with time, and consequently the stomata of detached leaves gradually recover until they finally behave more or less as those of attached leaves. It may be worth noting here that the increased rates of respiration which are often recorded as a result of wounding cells (Richards, 1896; Whiteman & Schomer, 1945; Saïd & El-Shishiny, 1947; and others), or even mechanical flexure without wounding (Audus, 1935), are also examples of 'Shock response'.

It is worth while to record that the time of appearance of the effect of severing varied according to the time at which the leaves were detached from their plants. Thus, when the stomata were normally widely open, and such opening was going to be maintained for a long time (Figs. 1 A and 2 A), then the stomatal response to the shock of severing lagged for at least 1 hr. behind the time of leaf detachment. On the other hand, when the stomatal opening was going to be maintained for only a short period, after which closing ensued (Figs. 1 B and 2 C), then the effect of severing appeared soon after detachment. It should be recalled that Oppenheimer & Mendel (1939) found that the closing of stomata of orange leaves started to take place 10 min. after leaf plucking.

These results are very interesting because they show that, in experiments where detached leaves are to be used, one must take into consideration the irregularities in the stomatal behaviour which may result from severing the leaves from their branches. In order that the effect of severing on the stomatal behaviour may be eliminated, the leaves should be left with their cut ends immersed in water (to prevent their wilting) until their stomata recover from the shock of severing and behave just like those of the attached leaves.

SUMMARY

Experiments are reported in which it was demonstrated that detachment of plant leaves from their branches may effect a shock stimulus, in response to which the stomata close. This response is most apparent when the stomata are normally tending to open or approaching their maximum openings. The effect of severing always lags behind the time of detachment, and the lag period differs according to the opening state of the stomata. The effect of severing usually lasts for a few hours, after which the stomata of both attached and detached leaves follow the same rhythmic course.

When the stomata of the attached leaves are normally closed, or tending to close, then detachment has very little or no apparent influence on the stomatal movements.

REFERENCES

Audus, L. J. (1935). Mechanical stimulation and respiration rate in the cherry laurel. *New Phytol.* **34**, 386.

Darwin, F. (1916). On the relation between transpiration and stomatal aperture. *Philos. Trans.* B, **207**, 413.

Darwin, F. & Pertz, D. F. M. (1911). On a new method of estimating the aperture of stomata. *Proc. Roy. Soc.* B, **84**, 136.

Desai, M. C. (1937). Effect of certain nutrient deficiencies on stomatal behaviour. *Plant Physiol.* **12**, 253.

Krause, H. (1935). Beiträge zur Kenntnis der Wasseraufnahme durch oberirdische Pflanzenorgane. *Öst. bot. Z.* **84**, 241.

Laidlaw, C. G. P. & Knight, R. C. (1916). A description of a recording porometer and a note on stomatal behaviour during wilting. *Ann. Bot., Lond.*, **30**, 47.

Lloyd, F. E. (1908). The physiology of stomata. *Pub. Carneg. Instn*, **82**.

Loftfield, J. V. G. (1921). The behaviour of stomata. *Pub. Carneg. Instn*, **314**.

Oppenheimer, H. R. & Mendel, K. (1939). Orange leaf transpiration under orchard conditions. *Bull. Agric. Res. Sta. Rehovot, Palestine*, no. 25.

Pinkhof, M. (1920). A new method of recording the modifications in aperture of stomata. *Proc. Kn. Akad. Wet. Amst.* **23**.

Richards, H. M. (1896). The respiration of wounded plants. *Ann. Bot., Lond.*, **10**, 531.

Saïd, H. & El-Shishiny, E. D. H. (1947). Respiration and nitrogen metabolism of whole and sliced radish roots with reference to the effect of alternation of air and nitrogen atmospheres. *Plant Physiol.* **22**, 452.

Whiteman, T. M. & Schomer, H. A. (1945). Respiration and internal gas content of injured sweet-potato roots. *Plant Physiol.* **20**, 171.

THE EFFECT OF STEM-RINGING ON THE DAILY MARCH OF STOMATAL RHYTHM IN *KALANCHOË* LEAVES

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(With 2 figures in the text)

It has often been observed that the amount of transpiration is less from the leaves of ringed stems. This reduction in transpiration seems to be largely due to the effects of ringing on the leaves and perhaps chiefly to the effect on the stomata. Thus Miss Pleasants* made a number of observations and found that the stomata on the leaves of ringed branches usually open only for a short time each day, or may show almost no opening at all, while those on normal branches open more widely and remain open for a longer time.

So far as the author is aware, no one studied, for prolonged periods, the effect of stem-ringing on the daily march of stomata. In the present investigation, the writer ringed the stems of potted *Kalanchoë marmorata* just above the soil surface and the ringed regions were completely filled with vaseline. Strips of epidermis were taken at intervals from leaves of ringed plants as well as from those of normal intact plants, fixed quickly in absolute alcohol and then the average widths of their stomata were determined by a standardized ocular micrometer. These observations began 2 hr. after ringing and were extended over 7 days. The strips were collected at 2 hr. intervals in the first day, at 4 hr. intervals in the second day and then at 6 hr. intervals in the following 5 days. The results of this experiment are graphically represented in Fig. 1.

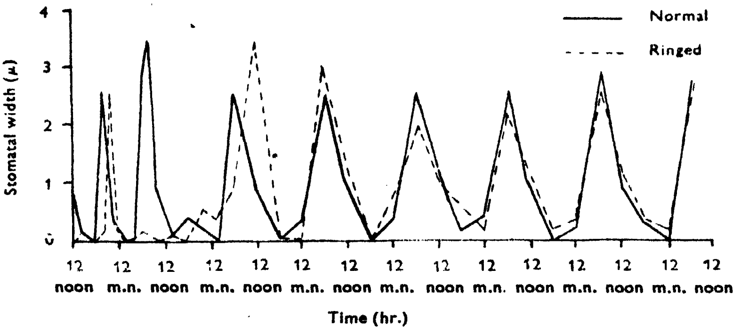

Fig. 1. Stomatal behaviour of leaves from ringed and normal plants during the week following ringing. Each point on the curves is the average of thirty determinations.

It is quite clear from the figure that for 2½ days after ringing, the stomata on leaves of ringed stems were apparently suffering from shock of ringing and behaved rather differently from those of normal plants. After 2½ days, the ringed plants seemed to have recovered from the shock of ringing and the behaviour of stomata on leaves of both ringed and normal plants was almost identical.

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* Quoted by O. F. Curtis in *The Translocation of Solutes in Plants*, McGraw-Hill Book Company, 1935.

Fig. 2 represents stomatal movements of both normal and ringed plants, studied 2 months after ringing of the stems. The figure clearly illustrates the complete recovery of the ringed plants from the shock caused by ringing the stems. This figure also shows unmistakable stomatal rhythmic movements during the 24 hr. of the day. Unpublished work indicates that this stomatal rhythm seems to be induced and predetermined by the daily repetition of day and night.

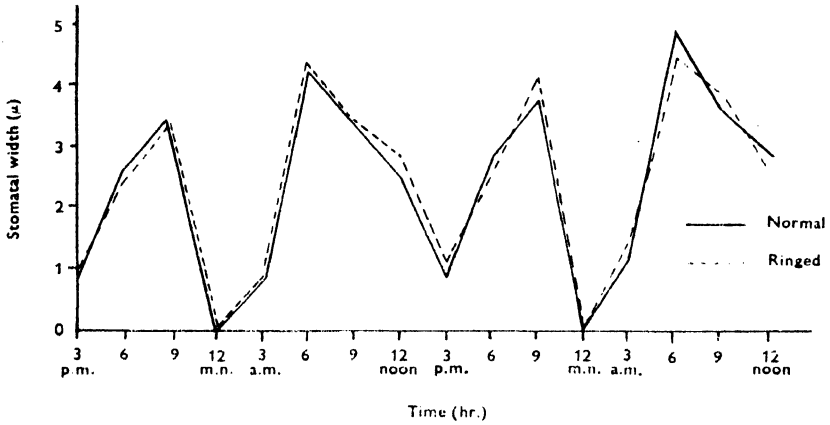


Fig. 2. Stomatal behaviour of leaves from ringed and normal plants, two months after ringing. Each point on the curves is the average of thirty determinations.

A NOTE ON A RAPID CLEARING TECHNIQUE OF WIDE APPLICATION

By KENNETH R. SPORNE, M.A., Ph.D.
The Botany School, University of Cambridge

(With Plate 5)

The use of lactophenol in conjunction with cotton blue staining, first described by Klebahn (1910), for demonstrating the presence of fungal hyphae in infected plant material is now a well-known technique, and there must be many mycologists who have noticed that under the clearing action of lactophenol plant tissues become almost transparent except for lignified xylem elements. It was this observation which led the writer, in 1939, to develop a rapid clearing technique, using pure lactic acid (B.P.) instead of lactophenol, for the morphological study of a wide range of botanical material. A method of clearing by means of lactic acid, which was similar to this in some respects, was described by Simpson (1929) and has since become widely used in America, though it does not appear to be well known in England. The technique described here was designed primarily for examining the internal vascular structure of the flower and has subsequently been found most useful for examining the arrangement of the sporangia within the sori of ferns; it has also been used to demonstrate the vascular bundles within the fertile spikes of members of the Ophioglossaceae and to show the arrangement of the sporangia within the cones of species of *Selaginella*. The principal advantages of the technique lie in its rapidity, in the fact that no staining is required, that it may be applied equally to fresh material, spirit material and dried herbarium specimens, and that preparations may be mounted permanently in the clearing fluid.

For studying the vascular structure of large or medium-sized flowers, longitudinal and transverse hand-cut slices of tissue up to 2 mm. in thickness may be used, while small flowers can often be examined whole, and for studying the contents of a fern sorus it is sufficient to use a portion of the frond without any preliminary dissection. Satisfactory results have been obtained with dried herbarium material, both of ferns and of angiospermous flowers, after soaking out by means of the modified Juel technique described by Tillson & Bamford (1938).

Before proceeding with the clearing process, it is necessary first to decolorize the tissues. Thus, if fresh material is used, all chlorophyll must be removed. Spirit material, though free from chlorophyll, is often coloured brown, and herbarium material usually contains a similar brown coloration. This can usually be removed by treatment with alkaline hydrogen peroxide ('20 vols.'), though the process may take 24 hr. or more. The clearing process consists of heating the material for a few minutes in pure lactic acid (B.P.) kept at 100° C. in a boiling water-bath. The preparation can then be examined in a watch-glass under a binocular microscope, and any dissection which may be required can then be carried out. Examination at this stage will also reveal any air bubbles which there may be within the tissues, and which must be removed under a vacuum pump before the preparation is mounted permanently.

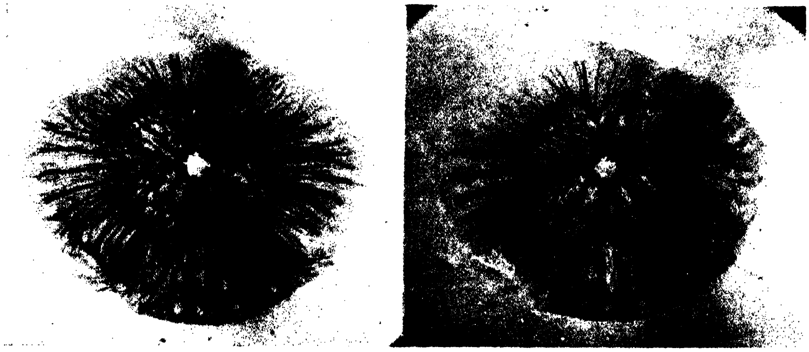


Fig. 1

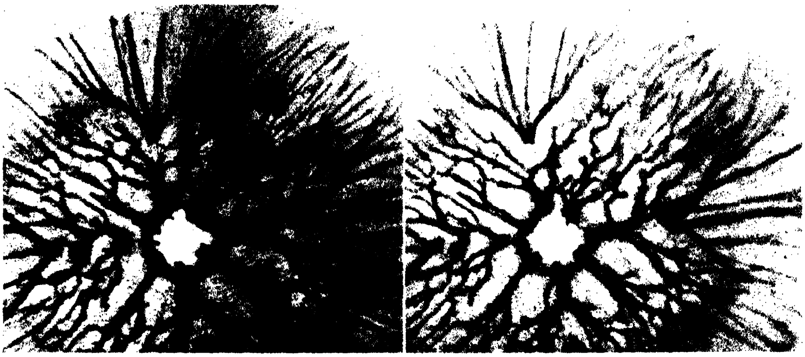


Fig. 2

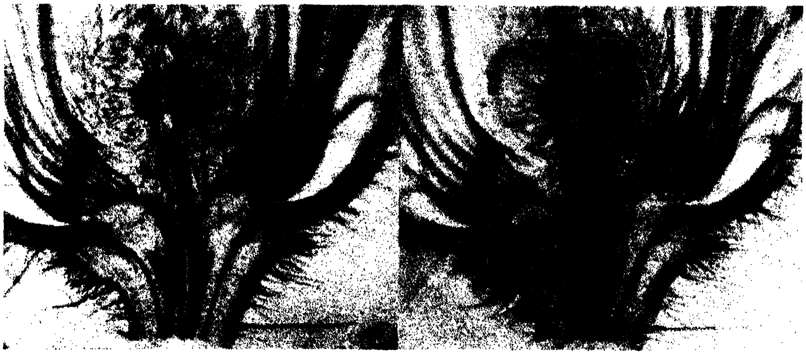


Fig. 3

SPORNE—A RAPID CLEARING TECHNIQUE OF WIDE APPLICATION

To make permanent mounts, a cell deep enough to hold the preparation should be built up on a microscope slide, and a useful cement for this purpose is one which has been used successfully in the Botany School, Cambridge, for many years in conjunction with lactophenol and cotton blue. It is resistant to lactic acid and consists of the following three constituents melted together:

Lanoline, anhydrous	4 parts,
Resin, powdered	8 parts,
Balsam, dry powder	1 part.

The cell can be constructed by applying successive layers of this cement by means of a hot iron rod. When sufficiently deep, it should be filled with lactic acid, the preparation should be placed in position and a cover-slip applied. The whole should be ringed with more of the cement and should be trimmed with a hot knife. The whole process, starting with good spirit material, does not take longer than 20 min., and preparations mounted in this way will keep for many years, provided that due precautions are taken. They should be stored horizontally in a cool place, since the cement is liable to become plastic in hot weather.

REFERENCES

KLEBAHN, H. (1910). Krankheiten des Sellerіes. *Z. PflKrankh.* **20**, 1.

SIMPSON, JENNIE L. S. (1929). A short method of clearing plant tissues for anatomical studies. *Stain Tech.* **4**, 131.

TILLSON, A. H. & BAMFORD, R. (1938). The floral anatomy of the Aurantioideae. *Amer. J. Bot.* **25**, 780.

EXPLANATION OF PLATE

Plate 5 consists of stereo-photo-micrographs, taken in 1948, of preparations mounted in 1939, of tetramerous flowers of *Sparmannia africana* (Tiliaceae). Fig. 1 is of a transverse slice through the receptacle, and shows how the stamen vascular supply arises from four epipetalous and four episepalous 'trunk-bundles'. Beneath the stamen bundles, the petal bundles can also be observed. Fig. 2 is of the same, more highly magnified. Fig. 3 is of a longitudinal slice, cut in the plane of the sepals, and shows the vascular supply to the sepals, the episepalous stamen groups and the carpels.

A NOTE ON CELL DIVISION IN THE DESMID *COSMARIUM MENEGHINII*

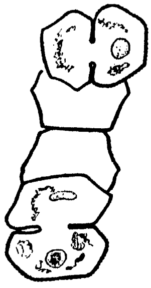
By J. M. THODAY

Department of Botany, Sheffield University

(With 4 figures in the text)

Cell division in *Cosmarium* and in related unicellular desmids has been described in some detail by Lütkemüller (1902, pp. 362 *et seq.*). Lütkemüller states that the separation of the daughter cells in these organisms is achieved by casting newly formed half-cell walls, but Fritsch (1935, p. 347) does not appear to regard this as fully established, since he states that the matter is worthy of fresh investigation.

During an examination of a culture containing dividing *Cosmarium meneghinii* a number of barely visible H-shaped membranes were observed (Fig. 1), and investigations of their origin disclosed that these were walls cast from the desmids in division. The drawings which were made at the time (1939) are published here, since they provide confirmation of Lütkemüller's findings. In division a new wall is formed between and around the new half-cells (Fig. 2), the adult half-cell wall is laid down within it, and the separation of the cells is achieved by casting the primary wall (Fig. 3). The convolutions of the surface of the secondary wall of the new half-cells were not visible at the time of separation. Fig. 4 shows a pair of dividing cells before the secondary wall has been formed in the young half-cells, stained in iodine and sulphuric acid. The swelling of the adult cell wall, absent in the young half-cells, is striking.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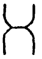
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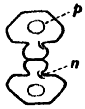

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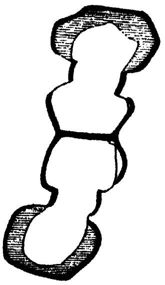
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Fig. 1. H-shaped membrane cast from the desmids during division.
Fig. 2. Formation of new half-cells: *n*, nucleus; *p*, pyrenoid.
Fig. 3. Separation of the developed daughter cells and the casting of the membrane.
Fig. 4. Dividing cells stained in iodine and sulphuric acid. Hatched areas blue, black walls unstained.

It is interesting to note that this method of separation of daughter cells is similar to the method of fragmentation in *Spirogyra nitida* (Fritsch, 1935, fig. 99H). It would seem reasonable to regard it as evidence of the secondary nature of the unicellular habit in these desmids. This might of course be held for all unicellular organisms with cell walls, which do not divide and separate within the parent cell wall as does *Chlamydomonas*.

The author wishes to express his indebtedness to the staff of the Department of Botany, the University College of North Wales, Bangor, for the culture in which these desmids were found, and for assistance in the identification of the species.

REFERENCES

FRITSCH, F. E. (1935). *The Structure and Reproduction of the Algae*, vol. 1. Cambridge.
LÜTKEMÜLLER, J. (1902). Die Zellmembran der Desmidaceen. *Beitr. Biol. Pfl.* **8**, 347–414.

REVIEWS

The Chemical Activities of Bacteria. By E. F. GALE, B.A., Ph.D. 5 × 7½ in., 200 pp. with 11 text-figures. London: University Tutorial Press, Ltd. 1947. Price 8*s.* 6*d.*

Because of their intense metabolism and the ease with which they can be handled under a wide range of controlled external conditions, bacteria are ideal organisms with which to investigate the fundamental chemical processes of life. The difficulties of breaking down complex reaction patterns into simpler individual stages are not so great as when larger organisms are used, and the properties of the enzymes which control the patterns are more easily studied. Also, the wide range of heterotrophic activity of bacteria offers endless scope and opportunity. Biochemists have for many years been exploiting this field intensively and their outstanding success has led to the appearance in recent years of a series of text-books on the subject of bacterial 'chemistry'. The subject has thus passed beyond the specialist stage to become a recognized branch of micro-biology—in other words, the research worker is now handing on his results to the student. Unfortunately, in this as in other fields, in doing so he often forgets the particular needs of the student, and there is a tendency for text-books to be written at research level and published at prices beyond the average student's pocket.

Dr Gale's book is intended to give an account of the chemical activities of bacteria suitable for students reading biochemistry, bacteriology and chemistry. He has succeeded: the book is inexpensive, comprehensive but concise, detailed without being too advanced. As a very large part of the book consists of general information about enzymes and enzyme action it should be of interest to a much wider public. Students of botany, who might perhaps claim an equal interest in these lowly plants and their physiological processes, will find the book of great use and it is recommended to them.

To describe the book as concise is perhaps an understatement. Dr Gale's command of written English has enabled him to condense his text almost to précis form without losing the reader's interest. An extremely wide field is covered and an enormous mass of information presented in a very limited space. No attention is paid to historical development, obsolete theory, controversy. Confining himself to what is established or generally accepted he sets out the current viewpoint in short, crisp, unambiguous prose. The limited references given are to review articles. As stated, most of the book is about enzymes—their nature, formation, mode of action, the enzymes and the reactions which they catalyse in carbon, nitrogen and phosphorus metabolism both inside and outside the cell. Dr Gale also discusses the overlapping subject of essential nutritional substances, including the 'vitamins'. Bacteria have proved a useful tool in elucidating the role of these substances in metabolism; now that this is known the substances can themselves be used as tools for further investigations and the scope of these investigations is described. The theoretical basis of chemotherapy and the pathological phenomena of virulence and toxin production are also discussed.

The attempt briefly to cover so large a field has its dangers. In places the book comes very near to being a catalogue of reactions which, one feels, are included for the sake of completeness. Personal prejudice suggests that certain aspects have received insufficient attention. Two chapters do not fit in very comfortably. The contents of chapter II on the nature and identification of bacteria do not seem necessary to the main theme. Chapter XII is designed to assist workers who wish to carry out investigations of bacterial metabolism without having access to the facilities of a bacteriological laboratory. Surely no one would be so unwise? Instead of the usual text-book instruction on elementary bacteriological technique (which is more fully available elsewhere) one would have liked, from such an acknowledged master, a few more tips on the tricks of the trade such as are not readily available; for instance, on how to rid a medium of all traces of a growth factor, or on filtration technique.

The book is designed for use; it is conveniently small, the binding is strong, the paper of very good quality, the printing delightfully clear and the formulae and diagrams very well managed in relation to the text.

G. METCALFE

Heredity and Variation in Micro-organisms. Cold Spring Harbor Symposia on Quantitative Biology. Vol. XI, 314 + xii pp., quarto, 183 figures (text + 14 plates). 1946. Published by the Biological Laboratory, Cold Spring Harbor, New York. Price $6.00 (postage extra).

Our knowledge of the gene and of the mechanism of evolution is largely based upon the study of complex organisms in which integrated processes of development come between a genotypic change and its phenotypic expression. We do not know the extent to which the knowledge so derived is applicable to simpler organisms and, though we conceive of evolution as occurring by the gradual selection and accumulation of minute hereditary changes in balanced genetic complexes, it is quite probable that simpler organisms may be able to accommodate relatively 'large' mutations. At present, therefore, we can only speculate upon the mutation rates possible and necessary to unicellular organisms with or without sexual reproduction, and to organisms whose genes are not arranged on chromosomes.

During the past few years a number of workers have turned to the study of variation in micro-organisms. The papers in this volume, originally presented at a meeting in 1946, describe many of their findings, and some of the discussion which then took place is included. Many of the twenty-seven papers are themselves in the nature of reviews, and it is only possible to mention a few points in the available space.

There are four papers describing work on viruses, eleven concerned with bacteria, seven with fungi and one with *Paramoecium*: of the remainder one is a discussion of variation in plastids, two describe variation in cancer cells and one is a discussion of the merits and limitations of the phase microscope. There is also an appendix which contains proposals concerning the nomenclature of nutritional types. Of necessity a fair amount of space is devoted to description and critical discussion of the experimental methods which have been and are being developed. The rapid multiplication rates, and the difficulties of isolating individual organisms lead to difficulties in distinguishing between variation and the selection of already existing variants. The choice of experimental material is often dictated by such considerations.

Hereditary variations in the viruses which attack *Escherichia coli* (Hershey, Delbrück & Bailey) affect, among other characters, their host range and the type of culture produced by the host. They are relatable to 'mutational sites' at each of which there appears to be a single structure subject to reversible alteration between two 'alleles'. Mutation at any one such site is independent of alteration at other sites. When a mixture of viruses infects a bacterium (only possible if the viruses are closely related) there may be an exchange of characters between them. For example, if a virus of type T_4 carrying the character r infects a bacterium together with type T_2r^+, T_4r^+ and T_2r may sometimes be recovered. These results are of the greatest interest since, as Hershey states, it is conceivable that among the viruses we may observe 'the operation of the principles of heredity at an elementary level of organization not far removed from that of the gene itself'. However, it should be borne in mind that these are relatively complex viruses. Care should also be taken before drawing too close an analogy between the phenomena described above and allelism and crossing over in classical genetics.

Variations which are hereditary and reverse with characteristic frequencies may be induced in a bacterium, *E. coli*, by the same mutagenic agencies—radiations and mustards—as produce gene mutations in higher organisms. Some of these variations lead to precisely the same biochemical requirements as do gene mutations in *Neurospora* (Tatum), but both these and mutations to phage resistance (Demerec & Latarjet) often do not manifest themselves until several cell generations after treatment, a fact which does not yet seem to admit of satisfactory explanation. While it seems certain that mutable factors exist in bacteria, it is evident, as Luria states, that the genetic systems involved are not necessarily similar to those of higher organisms. In any case all the papers on bacteria should be read with Dienes's discoveries and their possible implications in mind. He describes an unusual, but reproducible, method of reproduction in various bacteria. 'Large bodies' are formed which may give rise either to the normal forms, or to colonies of very tiny organisms. In *Proteus* these large bodies occur only at the zones of contact of colonies of two different strains: actual contact of cells is necessary but fusion does not occur. In a Bacteroides strain, on the other hand, it appears that two adjacent individuals take part in the formation of a large body. Dienes suggests the possibility of an exchange of characters analagous to the transformation of *Pneumococci*. The extract of an encapsulated strain of *Pneumococcus* can induce the formation of a capsule on an unencapsulated strain cultured in serum broth. The transformed *Pneumococcus* takes on, permanently, the type specificity of the strain from which the extract was made. The specific substance in the extract is apparently a desoxyribose nucleic acid, a fact with a bearing on the specificity of nucleic acids in general (McCarty *et al.*). The possibility that similar character changes may occur under normal conditions is suggested by the results of Lederburg & Tatum, who report that in mixed cultures of biochemical mutants of *Escherichia coli* reversions to the normal occur at rates which cannot be accounted for by the back mutation rates; the result is similar to that obtained after mixed infection of viruses, though the mechanisms need not necessarily be the same.

The work on fungi is on the whole more advanced because the tools of classical genetics may sometimes be used, though the situation is often complicated especially when the organisms are heterokaryons. Pontecorvo discusses genetic systems based on heterokaryosis at some length. A heterokaryotic system may provide some of the advantages of diploidy since, when different nuclei are present in the same cell, a gene in one may be dominant to a gene in another; the two nuclei may also provide each other's requirements, and combinations of characters may be achieved, in the absence of sex, by hyphal fusion. The possibilities of internuclear selection are especially important. In a cell which contains a number of each of two kinds of nucleus, each kind may produce substances necessary for the growth of the other. In such a situation, if one type of nucleus is in short supply it may not provide sufficient substrate for the growth of the other: within limits a balanced ratio of the two types of nucleus may be maintained. (It would seem possible that an analagous process of intergenic selection might provide some genetic stability in an organism without chromosomes or mitosis.)

Hollaender & Emmons report that sunlight can induce mutations in *Aspergillus*: ultra-violet components of the spectrum are involved.

Especial interest attaches to Spiegelman's work on adaptive enzymes in yeasts. The results seem similar to those of Sonneborn, whose work on cytoplasmic factors in *Paramoecium* reaches the quantitative stage in the experiments described here. The situation in yeast differs in that an external substance is involved. Strains of yeast occur capable of forming an enzyme in the presence of the substrate upon which that enzyme acts, and these differ in a gene from those incapable of forming the enzyme. The enzyme can only be formed *de novo* in the presence of both gene and substrate. However, provided that segregation occurs in the presence of the substrate, clones may be produced, lacking the gene, in which the enzyme either reproduces itself, or is reproduced by self-duplicating cytoplasmic factors, for as long as the culture continues to be supplied with substrate. In the absence of the substrate the enzyme is lost. Spiegelman discusses these results in relation to theories of enzyme production and gene control. No one interested in the problems of cell differentiation can fail to see the possibilities of a genetic character only developed in the presence of an extra-cellular chemical substance. Other papers in this volume will also be found to make interesting references to cell differentiation.

That the papers in this volume are of wide interest will be clear enough from these examples. The caution of the writers is generally commendable, though one doubts the wisdom of a mention of the word chromonema in a paper on bacteria, and feels the evidence for Lindegren's cytogene theory is not yet strong enough, as is evident in the discussion of his paper. But it is quite clear that anyone wishing to be up to date in his knowledge not only of cytogenetics, bacteriology and mycology, but also of biochemistry, enzymology and cell differentiation, will have to be familiar with the work of the authors of this most important book, and of those who follow in their footsteps.

J. M. THODAY

The Royal Botanical Expedition to New Spain. By HERBERT WILLIAM RICKETT. *Chronica Botanica*, vol. 11, no. 1, pp. 1–86, pls. 44–52. Waltham, Mass.: *Chronica Botanica* Co.; London: Wm. Dawson and Sons, Ltd. 1947. Price $2.50.

In 1787 Charles III of Spain sent an 'Expedición Botánico' to his American Dominion of Mexico 'to collect, identify and methodically describe the natural productions of the three kingdoms of Nature', to create a Botanic Garden and to found a Chair of Botany at the University. The history of this enterprise is contained in the National archives of Mexico and Dr H. W. Rickett, Bibliographer at the New York Botanic Garden, is to be congratulated on disinterring it from the contemporary files and presenting it as a consecutive story in a volume of *Chronica Botanica*. It is fortunate that these original files have survived to catch Dr Rickett's detached and critical eye. Had the history been written by a contemporary we should never, perhaps, have been able to enjoy the unvarnished tale of human strength and weakness, of official procrastination and individual initiative which the files reveal. From 1788 until 1820, when Spain lost her hold on Mexico, the story of the expedition revolves round the names of Sessé, Cervantes and Mociño, and it is remarkable how successfully they and their fellow-workers collected, described and classified the virtually unknown Flora of the country.

Dr Rickett's volume is well illustrated with photographs, maps and facsimiles of contemporary documents, and forms a really valuable contribution to botanical history. J. L. GILMOUR

Introduction to Carbohydrate Biochemistry. By D. J. BELL. 7 × 4¾ in. Pp. viii + 107. London: University Tutorial Press, Ltd. 2nd ed. 1947. Price 6*s*.

The second edition of this very useful little book will be welcomed in laboratories of plant physiology and biochemistry. It follows the lines of its predecessor, but has been thoroughly revised and brought right up to date. Some improvements of material and arrangement have also been made. The unsatisfactory first chapter on photosynthesis has been omitted and bacterial metabolism made over to Dr E. F. Gales's companion volume.

The organic chemistry of sugars and polysaccharides is usefully summarized. The oxygen glycosides, including the sap pigments, are summarily dispatched but the nucleotides receive more attention, together with phosphorylation and phosphorolysis. The last chapters are concerned with fermentation in yeast and glycolysis in muscle. Each chapter ends with a brief list of major references.

Unlike most second editions, this is actually shorter (by 5 pages) than the first. A total of 107 pages leaves no room for any alternative statements, and the very wide range of subjects treated is necessarily presented as a dogma. The dangers of this type of presentation in any biological subject needs no emphasis; but the young student for whom the book is intended should be warned. Admitting the difficulty, there can be no doubt as to the value of this unified presentation of carbohydrate biochemistry. The second venture appears to repeat the good features of the first and to avoid some of its blemishes. W. O. JAMES

The Living Plant. By I. V. Newman. 8½ × 5½ in. Pp. 128, with 6 half-tone illustrations. Wellington, New Zealand: A. H. and A. W. Reed. 1946. Price 8*s*. 6*d*.

Dr I. V. Newman's book is a laboratory hand-book for first-year University students of botany, and has been based upon the laboratory instruction sheets issued for the past few years to such students at Wellington College, New Zealand.

Dr Newman belongs to that category of teacher who finds himself thoroughly at home when beside a student at a laboratory bench, demonstrating methods and techniques which reveal the secrets of the structure and behaviour of that plant material, which through the years has not ceased to interest and excite him. The experience and enthusiasm of such teachers is invaluable, and much of this value is transmitted by this handbook. Students who follow its precise directions will be trained as careful manipulators and exact observers of the gross and microscopic structure of plants.

If this book represents fairly the full course of lectures and practical work for the first-year University student it is open to the charge that much too little regard is given to modern and experimental fields of botanical science. There is in places a teleological flavour in the interpretation of structures, and one notes with surprise that almost all the type material is derived from introduced European garden plants, so that the pea, bean, privet and so forth figure as largely as in the familiar parent works. *Fucus* has given place to *Hormosira*, and the families of flowering plants represent the New Zealand and Australian floras, but one cannot help regretting that the opportunity has not been taken of building the local botanical courses more thoroughly upon the local material. Not only would this be a gain in the interest of students, but it would tend to free them from the danger of repeated but often misleading generalization which arises from restricted and canalized observation. H. GODWIN

Hormones and Horticulture. The use of special chemicals in the control of plant growth. By George S. Avery and Elizabeth Bindloss Johnson, with the collaboration of Ruth M. Addoms and Betty F. Thomson. 6 × 9 in. Pp. xi + 326, with 51 figures in the text. New York and London: McGraw-Hill Publishing Co. Ltd. 1947. Price 27*s.*

Within the last twenty years, new horticultural methods, sprung from the results of fundamental research on plant-growth hormones, have, in the words of the authors, caused 'a real chemical revolution in agricultural practice'. It has been the purpose of the authors to collect and assess the practical value of all such published data on the use of special chemicals in the regulation of plant growth. The book is intended for those engaged in horticultural pursuits and for research workers in this or related fields.

The authors have succeeded in ushering into something like logical sequence the unwieldy and, for the most part, entirely empirically compiled mass of data accruing as a result of the intense applied research in this field over the last decade. The great bulk of these data is of American origin. Out of a total of seven hundred or so references made to original publications only about 25 % are from outside the U.S.A. This is probably a true reflexion of the extent of America's contribution to the practical aspect of the subject. It is gratifying to note that, in this remaining 25 %, important Russian work is adequately represented, especially in chap. x on the chemical production of new plant varieties. The subject treated covers all the practical aspects of plant growth and reproduction that have so far been found to be susceptible of control by low concentrations of special chemicals. The main subjects treated are the stimulation of the rooting of cuttings, control of fruit drop, fruit set, weeds and dormancy and the chemical production of new varieties by such agents as colchicine. The reviewer feels that the choice of title is unfortunate. Even if one concedes grudgingly the extension of the term hormone to include the wide range of purely synthetic growth-promoting substances such as 2:4-dichlorophenoxyacetic acid, one can scarcely allow the inclusion of ethylene chlorohydrin, tar oil distillate, boron or acenaphthene. Three out of the ten chapters deal almost exclusively with such compounds as these latter.

The greater part of the data is compiled in the form of concise tables of all relevant details of species, treatment, experimental conditions, plant reaction, author, etc. A third of the total text is occupied by these tables. In the text these tables are discussed mainly from the practical point of view, and precise instructions are given for all those treatments where applications of the relevant chemicals have been shown to be of economic advantage. The theory of action of the chemicals is mentioned only in so far as it is necessary for the practical grower to understand the principles on which the success of his treatment depends. To the ordinary student of botany, therefore, the book has little obvious value, except to illustrate the importance of fundamental knowledge as the basis of all advancement in the practical field.

Certain sections might well have been reduced, e.g. chap. vi, concerning the treatment of seeds to increase crop yield. Many pages are devoted to illustrating the fact that no conclusive stimulation by treatment with plant-growth substance has yet been demonstrated.

The book is well arranged and contains many clear and well-chosen illustrations, including some that are semi-diagrammatic and used to illustrate practical instructions. Where possible American trade names of the chemicals recommended are given. There is no doubt that the book will serve as a very useful source of information to both the practical horticulturalist and to the applied research worker.

L. J. AUDUS

Plant Physiology. By Meirion Thomas. 8½ × 5½ in. Pp. xi + 504, with 70 figures in the text. London: J. and A. Churchill, Ltd. 1947. Price 28*s.*

Since it was first published in 1935, this text-book of plant physiology has gained a wide popularity as a valuable account of the subject, especially suitable for the early stages of University work. Now in the 3rd edition the text has been fully revised with a considerable rearrangement and addition of new material.

The most extensive changes are to be found in the parts dealing with plant metabolism and related topics. Advantage is taken of the more complete account which can now be given of some important enzyme systems, notably those concerned with oxidation-reduction and fermentation processes. The discussions of photosynthesis, respiration, and metabolism of nitrogen are expanded to include more recent work, and a new chapter on the metabolism of dicarboxylic and tricarboxylic organic acids is added. The section dealing with growth and movement has been rearranged with a new chapter devoted to auxins and other growth substances. In the selection and treatment of the new material many of the valuable qualities of the earlier editions are maintained. Prof. Thomas has successfully taken a middle course between fact and theory; sufficient speculation is introduced to give interest and coherence to the general theme of physico-chemical interpretations of plant functions.

During the course of successive revisions of the book there has been an appreciable change in the balance between the different sections. The discussion of nutrition and metabolism, supported by the account of enzymes and by Appendices I and II, now occupies more than half of the text. In several of these parts the account is brought fully abreast with modern research and theory, such as, for example, in the treatment of the metabolism of organic acids, where important and hitherto unpublished data from the author's laboratory are introduced in the discussion of carbon dioxide fixation. On the other hand, the general water relations of plants, dealt with in Part II of the book, are considered at a relatively lower level; the shortcomings of the classical theories of the uptake of water and solutes by living cells are only briefly outlined, a treatment which may cause some disappointment, since work in the field seems to offer a good illustration of the close co-ordination of the activities in living cells.

In the hands of the student it is probable that the more advanced account of certain parts of the subject will lead to some difficulties and dangers. The formidable task of keeping the book in touch with modern work and ideas is apparent from the fact that several statements must already be considered as dated. For example, the occurrence of transaminase systems (p. 49) in plants is now fairly well established, and the specificity of proteolytic enzymes (p. 26) has been more fully defined. Such details clearly cannot be regarded as valid criticisms, but rather as illustrating the problems which confront the author in presenting the subject at this level.

In a text-book of this kind selection is inevitable, and the increased emphasis now placed on metabolic processes does undoubtedly reflect the growth of interest amongst plant physiologists in this direction. Owing to advances which have been made, it is here that the widest gaps have developed between the treatment of the subject in text-books and in monographs and reviews. Prof. Thomas has succeeded in bridging this gap at many points, and the new edition will be warmly and widely welcomed by plant physiologists. There is no doubt that the revision will further extend the usefulness of the book, and it may be confidently expected that many students will continue to derive great benefit from its stimulating outlook and clear statement of principles.

Despite the economies achieved by smaller type, the printing is pleasantly readable: a few misprints have survived proof reading, but they are sufficiently obvious not to cause any serious difficulty.

E. W. YEMM

The Genetics of Garden Plants. By M. B. CRANE and W. J. C. LAWRENCE, with a foreword by SIR DANIEL HALL. 9 × 6 in. Pp. 299, with 67 figs. and illustrations and 44 tables. London: Macmillan & Co. Ltd. 3rd ed. 1947. Price 16*s*.

The third edition of this work is enlarged by the inclusion of results which have been published since the appearance of the previous edition. The aim of the authors has been two-fold: first to provide an introduction to the principles of genetics and cytology, an aim which has been achieved clearly and concisely in the first three chapters, and secondly to give an account of recent experimental work in relation to horticulture, to which the remainder of the book is devoted.

The fundamental principles of genetics and chromosome behaviour, which are not always easily understood without reference to well-established examples, are amply illustrated by examples discussed in the second part of the work. These include a large range of the commonly cultivated plants of the flower- and vegetable-garden, with a detailed but concise account of the history of cultivation of each species and also of the present state of knowledge of its genetics, with alternative opinions where the ideas of different workers are not in agreement. The last five chapters are concerned with certain aspects of genetics which are of particular interest to the horticulturist: bud-sports and chimaeras; incompatibility, including an account of the authors' own work on cherries, plums, pears and apples, with particular emphasis on the importance to fruit-growers of interplanting compatible varieties; sterility; xenia (the influence of foreign pollen on the maternal tissue), which is discussed in greater detail than in earlier editions; and the origin of new and improved forms.

Only two minor points call for criticism: on p. 155 there is unnecessary repetition in listing the interspecific crosses made in *Prunus*; and on p. 186 the quotation from East (1929) concerning the frequency of occurrence of incompatibility of pollen and style in flowering plants is misleading, since East's more recent survey (1940) of the distribution of incompatibility reveals that his earlier figures included incompatibility associated with heterostyly.

This book can be recommended to all who are concerned with horticultural practice, and also to students of genetics and evolution since, as Darwin originally emphasized, the artificial selection by man of certain characters, in the breeding of plants and animals, provides valuable information which may throw light on the influence of natural selection in wild organisms.

H. L. K. WHITEHOUSE

Plant Viruses. By KENNETH M. SMITH, F.R.S. 7 × 4½ in. Pp. ix + 78, with 8 plates and 3 text illustrations. London: Methuen & Co. Ltd. 2nd ed. 1948. Price 6*s*.

This volume of the well-known 'Methuen's Monographs' series on biological subjects has been entirely rewritten since its original appearance in 1935. Now based on a short course of lectures given annually in the Botany School at Cambridge, 'it is intended for students without previous knowledge of viruses who wish to know the broad outlines of the work in this particular field'. There are eight chapters, of which the first deals with the history of the subject, and with its economic importance so far as Great Britain is concerned. It seems a pity to have shortened this second part of the chapter so much as compared with the first edition: some mention of the chief plant virus problems of other countries, and particularly of the tropics, might have been retained. The second chapter, the longest in the book, deals with the virus as it affects the plant, including the question of virus strains and immunity—more conveniently considered here than in relation to the serology of viruses, as formerly. Serology is often a stumbling block for the botanist, who will be grateful for the clear exposition of its principles and terminology in a later chapter dealing also with the classification of viruses. The third and fourth chapters cover modes of transmission, natural and artificial, and the relationship with insect vectors. Again, the treatment is a little too condensed. The ecological aspects of spread in the field, to which about six pages were devoted in the original edition, and on which much new work has since been published, are omitted entirely, and the differences between the 'persistent' and 'non-persistent' types of virus might well have been further elaborated—as in some of the author's own recent papers—with reference to rates of inactivation within the insect. The ensuing chapter, on the isolation and properties of the viruses themselves, is excellent, a minor criticism being the misleading wave-length scale on the right of Fig. 2, which suggests a linear virus particle. After serology and classification, already noted, the control of plant virus diseases is next dealt with under the five headings of elimination of the sources of infection, avoidance of and direct attack on the insect vectors, breeding resistant varieties, and cure of infected plants. For completeness, an additional category would be needed for indirect measures, chiefly cultural, designed to mitigate the effects of virus diseases for which control by any of the above-mentioned has proved inadequate. The final chapter, on the nature of viruses, reviews impartially the various theories which have been put forward to account for the origin of these fascinating entities, though it is not always clear whether the discussion is intended to include the animal viruses, or not. The book has a very useful bibliography of 102 titles, and an adequate index. A feature of the new edition is the inclusion of photographic illustrations, of which the most striking are the magnificent electron micrographs of tobacco mosaic and tomato bushy stunt viruses, after Williams and Wyckoff. With the text considerably shortened, however, the increased price of the book appears excessive even for present circumstances. The impression remains that the original edition was both better value for the money, and a better introduction to the subject as it then stood. Nevertheless, the present edition deserves to be read by all students of biology, and will be welcomed by those workers in other subjects who wish to keep abreast of developments in this rapidly expanding field.

T. E. T. BOND

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THE
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EDITED BY
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INDEX OF AUTHORS

PAGE

Ainsworth, G. C. Biology of Pathogenic Fungi (Review) 124

Allison, J. and Godwin, H. Bronze Age plant remains from Wiltshire. Data for the study of postglacial history. XII 253

Alston, A. H. G. Genera Filicum—The Genera of Ferns (Review) 278

Ashby, E., Wangermann, E. and Winter, E. J. Studies in the morphogenesis of leaves. III. Preliminary observations on vegetative growth in *Lemna minor* 374

Audus, L. J. Studies on the *p*H relationships of root growth and its inhibition by 2:4-dichlorophenoxyacetic acid and coumarin 97

Beevers, H. (*see* Thomas, M.)

Böcher, T. W. Racial divergences in *Prunella vulgaris* in relation to habitat and climate 285

Bond, T. E. T. The Diseases of the Flax Plant (*Linum usitatissimum* Linn.) (Review) . 122

—— (1) Diseases of Cereals. (2) Diseases of Potatoes, Sugar Beet and Legumes (Review) 281

—— British Mycological Society, Proceedings of the Jubilee Meeting held in London, 20–25 October 1946 (Review) 461

Chakravarty, H. L. Morphology of the staminate flower of Bottle Gourd, *Lagenaria leucantha* (Duch.) Rushby 448

Clapham, A. R. British Flowering Plants. Evolution and Classification of Families and Genera with Notes on their Distribution (Review) 281

Conway, V. M. Postglacial Forest Succession, Climate and Chronology in the Pacific Northwest (Review) 119

Corner, E. J. H. The Annonaceous seed and its four integuments 332

—— Flora Malesiana. Series I, vol. 4, Spermatophyta, part 1 (Review) . . . 462

Editor. Genetics in Swedish Forestry Practice (Review) 282

Emmelin, N. and Feldberg, W. Distribution of acetylcholine and histamine in nettle plants 143

Feldberg, W. (*see* Emmelin, N.)

Gilmour, J. S. L. (*see* Peace, T. R.)

Gilson, M. R. Uredineana. Receuil d'Études Systématiques et Biologiques sur les Urédinées du Globe (Review) 119

Godwin, H. (*see* Allison, J.)

—— An Introduction to Plant Anatomy (Review) 282

Gregory, F. G. Vernalization and Photoperiodism. A Symposium (Review) . . . 461

Hanly, V. F. (*see* Turner, J. S.)

Haque, Ashraful (*see* Maheshwari, P.)

Heath, O. V. S. Studies in Stomatal Behaviour. II. The Role of Starch in the light response of stomata. Part 1. Review of literature, and experiments on the relation between aperture and starch content in the stomata of *Pelargonium zonale* . . 186

PAGE

Hope-Simpson, J. F. The Grasslands of Latin America (Review) 122

James, W. O. The amino-acid precursors of the belladonna alkaloids 172

James, W. O. Lectures on the inorganic nutrition of plants (Review) 118

—— Submicroscopic morphology of protoplasm and its derivatives (Review) 118

Jane, F. W. A Manual of the Timbers of the World (Review) 280

Jones, E. W. Der Lindenmischwald (Tilieto-Asperuletum taurinae) des schweizerischen voralpinen Föhn- und Seenbezirkes, seine pflanzensoziologische und forstliche Bedeutung (Review) 121

—— Botany of the Canadian Eastern Arctic. Part II. Thallophyta and Bryophyta. (Nicholas Polunin). E. W. Jones 279

Lerner, H. J. A note on the life history of *Splachnidium rugosum* L. (Grev.) 277

Lund, J. W. G. New or rare British Chrysophyceae 453

McKee, H. S. Review of recent work on nitrogen metabolism 1

Maheshwari, P. and Haque, Ashraful. The embryo sac of *Chrysanthemum parthenium* 255

Meeuse, B. J. D. Observations on the enzymatic action of maple and birch saps 125

Parker-Rhodes, A. F. The Basidiomycetes of Skokholm Island. II. Genetical implications of spore measurements in two agarics 382

Peace, T. R. and Gilmour, J. S. L. The effect of picking on the flowering of Bluebell, *Scilla non-scripta* 115

Reid, Mrs E. M. The Late Glacial flora of the Lea Valley 245

Sporne, K. R. A new approach to the problem of the primitive flower 259

Street, H. E. Experimental methods available for the study of the nitrogen metabolism of plants. A review of some recent advances 84

Subramanyam, K. An embryological study of *Lobelia pyramidalis* Wall., with special reference to the mechanism of nutrition of the embryo in the family Lobeliaceae 365

Thomas, H. and Beevers, H. Physiological studies on acid metabolism in green plants. II. Evidence of CO_2 fixation in *Bryophyllum* and the study of diurnal variation of acidity in this genus 421

Thomas, M. Physiological studies on acid metabolism in green plants. I. CO_2 fixation and CO_2 liberation in Crassulacean acid metabolism 390

Turner, J. S. and Hanly, V. F. Succinate and plant respiration 149

Wangermann, E. (*see* Ashby, E.)

Whitehouse, H. L. K. Multiple-allelomorph heterothallism in the fungi 212

—— Svalöf 1886–1948: History and Present Problems (Review) 282

Winter, E. J. (*see* Ashby, E.)

Yemm, E. W. Glutamine in the metabolism of barley plants 315

INDEX OF SUBJECTS

PAGE

Acer spp., Enzymatic action of saps of—B. J. D. Meeuse 125

Acetylcholine, Distribution of, in nettle plants—N. Emmelin and W. Feldberg 143

Acid metabolism in green plants, Physiological studies on. I. CO_2 fixation and CO_2 liberation in Crassulacean acid metabolism—M. Thomas 390

Acid metabolism in green plants, Physiological studies on. II. Evidence of CO_2 fixation in *Bryophyllum*, etc.—M. Thomas and H. Beevers 421

Alkaloids, of belladonna, The amino-acid precursors of—W. O. James 172

Amino-acid precursors of the belladonna alkaloids—W. O. James 172

Annonaceae, Seeds of, and their four integuments—E. J. H. Corner 332

Atropa belladonna, The amino-acid precursors of the alkaloids of—W. O. James 172

Barley, Glutamine in the metabolism of—E. W. Yemm 315

Basidiomycetes of Skokholm Island. II. Genetical implications—A. F. Parker-Rhodes 382

Betula spp. Enzymatic action of saps of—B. J. D. Meeuse 125

Bronze Age plant remains from Wiltshire—J. Allison and H. Godwin 253

Bryophyllum, Evidence of CO_2 fixation in, and diurnal variation of acidity in—M. Thomas and H. Beevers 421

Chrysanthemum parthenium, The embryo-sac of—P. Maheshwari and Ashraful Haque 255

Chrysophyceae, New or rare British—J. W. G. Lund 453

CO_2 fixation and CO_2 liberation in Crassulacean acid metabolism—M. Thomas 390

CO_2 fixation, Evidence of, in *Bryophyllum*—M. Thomas and H. Beevers 421

Coumarin, Inhibition of root growth by—L. J. Audus 97

2:4 Dichlorophenoxyacetic acid, Inhibition of root growth by—L. J. Audus 97

Diurnal variation of acidity in *Bryophyllum*—M. Thomas and H. Beevers 421

Embryology of *Lobelia pyramidalis*—K. Subramanyam 364

Embryo-sac of *Chrysanthemum parthenium*—P. Maheshwari and Ashraful Haque 255

Enzymatic action of maple and birch saps—B. J. D. Meeuse 125

Experimental methods, for the study of the nitrogen metabolism of plants—H. E. Street 84

Flower, the primitive, A new approach to the problem of—K. R. Sporne 259

Fungi, Multiple-allelomorph heterothallism in—H. L. K. Whitehouse 212

Glutamine, in the metabolism of barley plants—E. W. Yemm 315

Heterothallism, Multiple-allelomorph, in the fungi—H. L. K. Whitehouse 212

Histamine, Distribution of, in nettle plants—N. Emmelin and W. Feldberg 143

Inhibition of root growth by 2:4 dichlorophenoxyacetic acid and coumarin—L. J. Audus 97

Integuments of the annonaceous seed—E. J. H. Corner 332

Lagenaria leucantha, morphology of the staminate flower of—H. L. Chakravarty 448

PAGE

Late glacial flora of the Lea Valley—Mrs E. M. Reid 245

Lea Valley, The late glacial flora of—Mrs E. M. Reid 245

Leaves, Studies in the morphogenesis of. III. Preliminary observations on vegetative growth in *Lemnor minor*—E. Ashby, E. Wangermann and E. J. Winter 374

Lemna minor, Preliminary observations on vegetative growth in—E. Ashby, E. Wangermann and E. J. Winter 374

Lobeliaceae, Mechanism of nutrition of the embryo in—K. Subramanyam 365

Lobelia pyramidalis, An embryological study of—K. Subramanyam 365

Morphogenesis of leaves, Studies in the. II. Preliminary observations on vegetative growth in *Lemna minor*—E. Ashby, E. Wangermann and E. J. Winter 374

Morphology of the staminate flower of Bottle Gourd, *Lagenaria leucantha* (Duch.) Rushby—H. L. Chakravarty 448

Nitrogen metabolism, Experimental methods available for the study of—H. E. Street . 84

Nitrogen metabolism, Review of recent work on—H. S. McKee 1

Pelargonium zonale, Review of literature, and experiments on the relation between aperture and starch content in the stomata of—O. V. S. Heath 186

*p*H relationships of root growth—L. J. Audus 97

Picking, The effect of, on the flowering of *Scilla non-scripta*—T. R. Peace and J. S. L. Gilmour 115

Postglacial history, Data for the study of. XII. Bronze Age remains from Wiltshire—J. Allison and H. Godwin 253

Prunella vulgaris, Racial divergences in, in relation to habitat and climate—T. W. Böcher 285

Races of *Prunella vulgaris* in relation to habitat and climate—T. W. Böcher . . . 285

Respiration, Succinate and, in plants—J. S. Turner and V. F. Hanly 149

Root growth, Studies on the *p*H relationships of, and inhibition by 2:4 dichlorophenoxyacetic acid and coumarin—L. J. Audus 97

Scilla non-scripta, Effect of picking on the flowering of—T. R. Peace and J. S. L. Gilmour 115

Skokholm Island, Basidiomycetes of—A. Parker-Rhodes 382

Splachnidium rugosum, A note on the life-history of—N. H. Lerner 277

Spore measurements, Genetical implications of, in two agarics—A. F. Parker-Rhodes . 382

Succinate and plant respiration—J. S. Turner and V. F. Hanly 149

Starch, The role of, in the light response of stomata—O. V. S. Heath 186

Stomata, The role of starch in the light response of—O. V. S. Heath 186

Urtica spp., Distribution of acetylcholine and histamine in—N. Emmelin and W. Feldberg 143

Vegetative growth, Preliminary observations on, in *Lemna minor*—E. Ashby, E. Wangermann and E. J. Winter 374

REVIEWS: PAGE

A Manual of the Timbers of the World (Alexander L. Howard)—F. W. Jane 280

An Introduction to Plant Anatomy (A. J. Eames and L. J. MacDaniels)—H. Godwin 282

Biology of Pathogenic Fungi (Edited by Walter J. Nickerson, with a Foreword by J. G. Hopkins)—G. C. Ainsworth 124

Botany of the Canadian Eastern Arctic. Part II. Thallophyta and Bryophyta (Nicholas Polunin)—E. W. Jones 279

British Flowering Plants. Evolution and Classification of Families and Genera with Notes on their Distribution (John Hutchinson)—A. R. Clapham 281

British Mycological Society, Proceedings of the Jubilee Meeting held in London, 20–25 October 1946—T. E. T. Bond 461

Der Lindenmischwald (Tilieto-Asperuletum taurinae) des schweizerischen voralpinen Föhn- und Seenbezirkes, seine pflanzensoziologische und forstliche Bedeutung (Dr Walter Trepp)—E. W. Jones 121

(1) Diseases of Cereals. (2) Diseases of Potatoes, Sugar Beet and Legumes (W. A. R. Dillon Weston)—T. E. T. Bond 281

Diseases of the Flax Plant (*Linum usitatissimum* Linn.) (Arthur E. Muskett and John Colhoun)—T. E. T. Bond 122

Flora Malesiana. Series I, vol. 4, Spermatophyta, part 1 (General Editor: Dr C. G. G. J. Van Steenis)—E. J. H. Corner 462

Genera Filicum—The Genera of Ferns (E. B. Copeland)—A. H. G. Alston 278

Genetics in Swedish Forestry Practice (Bertil Lindquist)—Editor 282

Grasslands of Latin America (G. M. Roseveare)—J. F. Hope-Simpson 122

Lectures on the Inorganic Nutrition of Plants (D. R. Hoagland)—W. O. James 118

Postglacial Forest Succession, Climate and Chronology in the Pacific Northwest (Henry P. Hansen)—V. M. Conway 119

Submicroscopic Morphology of Protoplasm and its Derivatives (A. Frey-Wyssling)—W. O. James 118

Svalöf 1886–1946: History and Present Problems (Edited by A. Akerman, O. Tedin and K. Fröier. English Technical Editor, R. O. Whyte)—H. L. K. Whitehouse 282

Uredineana. Recueil d'Études Systématiques et Biologiques sur les Urédinées du Globe (Edited by A. L. Guyot)—M. R. Gilson 119

Vernalization and Photoperiodism. A Symposium (A. E. Murneek, R. O. Whyte *et al.*)—F. G. Gregory 461

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REVIEW OF RECENT WORK ON NITROGEN METABOLISM

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(With 15 Figures in the text)

CONTENTS

	PAGE
Introduction	1
Methods	2
Assimilation of nitrogen by plants	
(*a*) Inorganic nitrogen	4
(*b*) Relations between assimilation of nitrogen and of phosphorus	8
(*c*) Organic nitrogen	8
(*d*) Nitrogen fixation	9
Losses of nitrogen from plants	17
Miscellaneous effects of nitrogen supply on plants	20
Metabolic significance of organic acids	21
Transamination	25
Synthesis of amino-acids	28
Amide metabolism	29
Metabolism of amino-acids	32
Proteins and their synthesis	36
Alkaloids	46
Metabolic relations of urea	57
Purine metabolism	58
Cyanide metabolism	58
Conclusion	59
References	59

INTRODUCTION

The main purpose of this review is to discuss work, published in the period 1937–47, on nitrogen metabolism in plants. An earlier review (McKee, 1937) is taken as a starting point, but work published before 1937 is included when this seems useful. Papers seen in abstract only or quoted from other authors are so specified in the references, but not necessarily in the text. It is recognized that reference to papers not actually seen is undesirable *per se*; it may, however, be preferable to omitting data unavailable to the reviewer in the original. Such references are avoided where possible, and form only a small proportion of the total.

A rigid definition of the period to be covered is impracticable; the boundaries also of the subject cannot be stated precisely. The co-ordination and integration of the different metabolic activities of living matter have long been recognized, and it will be seen later that knowledge of the interlocking systems maintaining this integration, though still rudimentary, has increased during the period under review. These interactions make discussion of any particular metabolic system as a closed unit inadequate. Relations of nitrogen metabolism to other metabolic processes are therefore considered in some detail.

No claim is made that all relevant papers have been consulted. Such a claim would, indeed, be rendered meaningless by the arbitrary boundaries of the subject. It is hoped, however, that the papers listed will provide a basis for more complete study of any particular point. Some papers have been arbitrarily excluded as appearing not to provide new information, some seen only in abstracts too brief to indicate their true value, and some are sure to have escaped notice. No clearly relevant paper has been deliberately excluded. Few papers are completely devoid of useful information, though the tendency, due to desire for rapid communication of results or perhaps to considerations of priority, to publish in several short papers data which might well make a single paper of moderate size, is to be deplored, at least by all using bibliographies.

Although the main emphasis is on the metabolism of green plants, much work on other organisms, especially animals, is considered. This is due to the known metabolic similarities in organisms of very varied types, and to the more complete study of many metabolic systems with animal than with plant material. Results with one species, let alone larger aggregates, cannot, however, be more than illustrative for another. Some metabolic systems are known from a very wide range of organisms, but others may be confined to single species, or, indeed, to single individuals.

METHODS

(*a*) *Analytical methods.* Analytical methods, which ultimately define the advances possible to metabolic research, have been considerably improved during the period under review. It is proposed to mention some of the more important work, but not to discuss the field in any detail.

The main advances have been in the determination of individual amino-acids. There has been a great development of microbiological methods, using a test organism requiring the substance being determined and comparing some criterion of its growth in a medium where the content is known and in one containing the unknown sample. The use of such methods is reviewed by Snell (1946).

Other promising recent developments are chromatographic methods for identification and determination of amino-acids in complex mixtures (Consden, Gordon & Martin, 1944; Tristram, 1946), and the isotope dilution method. In the latter (reviewed by Shemin & Foster, 1946) a sample of the amino-acid to be estimated, containing an excess of isotopic nitrogen, is added to the mixture being analysed. Its content in the analytical sample is then given by

$$B = A(C_0/C - 1),$$

where C_0 and C are the values of excess of isotopic nitrogen in the compound as added and as isolated. Quantitative isolation is not required, but the accuracy of the method depends on the purity of the compound finally isolated. The method is not one for routine analysis but may be of great value in checking simpler methods. Important papers on the determination of individual amino-acids or special groups are: Bailey, Chibnall, Rees & Williams (1943) (dicarboxylic amino-acids); Cohen (1939*a*) (glutamic acid); Chibnall, Rees & Williams (1943*b*) dicarboxylic and basic amino-acids); Macpherson (1946) (basic amino-acids); Rees (1946) (threonine and serine). The determination by Brand, Saidel, Goldwater, Kassel & Ryan (1945) of the amino-acid composition of β-lactoglobulin is a noteworthy achievement in protein analysis. They give the composition, in terms of amino-acid residues, as: glycine 8, alanine 29, valine 21, leucine 50, isoleucine 27, proline 15, phenylalanine 9, cysteine 4, cystine 8, methionine 9, tryptophane 4, arginine 7, histidine 4, lysine 33, aspartic acid 36, glutamic acid 24, glutamine 32, serine 20, threonine 21, tyrosine 9. It should be noted that all ammonia produced on hydrolysis has arbitrarily been assigned to glutamine; presence of asparagine in the protein molecule is not excluded.

The fundamental problems in amino-acid analysis of proteins are discussed by Chibnall

(1946) and Lugg (1947*a*). Analysis of amino-acids in a protein involves preliminary hydrolysis, a step whose possible effects on the amino-acids liberated have led to much uncertainty. Acid, alkaline and enzymatic hydrolysis have all been used. The observation (Steinhardt & Fugitt, 1942) that organic acids (e.g. cetylsulphonic, dodecylsulphonic) of high molecular weight hydrolyse peptide and amide bonds at comparatively mild temperatures may provide a useful alternative to the more widely used methods. Chibnall, Rees & Williams (1943*a*) discuss in a salutary paper insidious errors that can occur in the apparently simple determination of total nitrogen.

The importance of isotopic tracers, especially C^{13} and N^{15}, in recent metabolic work will be obvious to the reader and need not be stressed here. Their use is considered in detail by Kamen (1947*a*, *b*).

(*b*) *Deductive methods in determination of metabolic pathways.* An intermediate product in a series of reactions whose rates are balanced will not accumulate unless the system is blocked in some way to prevent its breakdown. Metabolites involved in a complex set of interactions may therefore play important parts without ever being present in amounts large enough to be isolated.

Three main methods have been used to block reactions to permit accumulation and identification of intermediates: (1) specific poisoning, using reagents such as iodoacetate or malonate, whose action is comparatively specific for some step in the reaction sequence; (2) removal by chemical means of an intermediate, causing a shift of equilibrium favouring its continued formation; (3) separation of catalytic systems in cell-free systems. Each of these methods has advanced knowledge of intermediate metabolism, especially of catabolic processes. Their limitations are, however, clear. Even when one of these methods appears to block only a single reaction, the possibility of other undesired, and perhaps unrecognized, effects must be considered. Method (2), the 'Abfangmethode', involves the danger of artificially increasing the rate of a side reaction and so exaggerating its importance.

Another method of studying intermediate metabolism has already had its initial successes and promises considerable future development. Its rise was forecast by Haldane (1937), who wrote: 'the new branch of biochemistry which will, I believe, arise from genetics will be concerned largely with the stages of synthesis of such molecules as chlorophyll and cyanin.' The method has been developed mainly by Beadle and his co-workers, using mutant strains, produced by irradiation, of the mould *Neurospora crassa*. It has in some cases been possible to isolate several strains, all incapable, as shown by abnormal nutrient requirements, of synthesizing substances found in the normal type, but genetically and biochemically distinct. When different mutants show varied responses to putative intermediates in the synthesis of the substance they lack, it is possible to determine the point at which synthesis is blocked in each, and to deduce the course of synthesis in the normal organism. This work is reviewed by Beadle (1945); its application to the synthesis of choline (Horowitz, 1946) and of methionine (Horowitz, 1947) is discussed on p. 35. Horowitz, Bonner, Mitchell, Tatum & Beadle (1945) report a mutant strain unable to synthesize adenine and accumulating a purple pigment, apparently a polymer of a purine whose conversion to adenine occurs normally but is here blocked. A similar accumulation of cystathionine occurs in a mutant which can form but not metabolize it (see p. 35). A somewhat similar approach to biochemical problems is provided by comparison of the metabolic products of related species. This has not been widely used, though some data for it would appear to be available.

Accumulation of a given metabolite may thus be considered due to a lack of balance between its formation and utilization, or even to an 'error of metabolism' in the tissue concerned. In some species certain phases of metabolism seem out of balance. The distribution of ascorbic acid, better known than that of most plant constituents owing to its importance in human nutrition and to the comparative reliability of a simple method for its estimation, may serve as an example. It occurs in practically all plant tissues tested, with occasional striking accumulations, notably in leaves of *Gladiolus*, *Iris*, *Citrus*, *Tropaeolum* and parsley (*Petroselinum sativum*) and fruits of *Rosa* spp., *Juglans* spp., *Actinidia kolomicta* and *Psidium guajava*. Its metabolism in the plant is essentially unknown, but this accumulation, in widely scattered species and in such varied organs as active leaves and the fleshy pericarps of fruits, to levels far higher than in the great majority of species, suggests derangement of the balance between its formation and utilization. Rubin, Artsikhovskaya & Proskurnikova (1946) state that most of the ascorbic acid of rose fruits takes no part in the respiratory processes. Similar arguments may be applied to accumulation of carotene in carrot (*Daucus carota*) roots, and perhaps to accumulation of carbohydrate in some 'storage' organs. Archbold (1945) states that sugar accumulated in barley stems seems unavailable for growth and development, and points out that tops of fleshy roots, e.g. carrot, sugar beet, and buds of tubers of potato and Jerusalem artichoke (*Helianthus tuberosus*), can develop new plants after removal of most of the storage tissue.

ASSIMILATION OF NITROGEN BY PLANTS

(*a*) *Inorganic nitrogen*

The activities of soil bacteria ensure that both ammonium and nitrate are available to plants in natural conditions. Most plants seem able to use both, but some are better adapted to using oxidized or reduced nitrogen. The oxido-reduction properties of ammonium and nitrate should be considered in comparing them as nitrogen sources for plants (see p. 19). In agricultural practice ammonium is generally supplied as the sulphate. This fortuitous association of increased sulphur supply with ammonium complicates comparison of the latter with nitrate.

Barley, oat and rye seedlings (Arenz, 1941; Kappen & Wienhues, 1942) grow better with nitrate than with ammonium, as do some other cereals (Burström, 1945) and sugar beet (Vlasyuk, 1940*a*, *b*). Bonner (1946) confirms earlier reports that rice uses ammonium better than nitrate. Several planktonic algae studied by Chu (1942) grew equally well with ammonium or nitrate at high but better with nitrate at low levels of nitrogen supply. Isolated potato shoots grow better with ammonium than nitrate; nitrate utilization seems an important bottleneck in protein synthesis (Street, Kenyon & Watson, 1946*a*). Weeds are often stated to require nitrate, but Marthaler (1937) found that *Chenopodium album* makes very poor growth on nitrate alone and does much better with ammonium, though often absorbing and storing large amounts of nitrate which it cannot use. Other weeds may grow better with nitrate but use ammonium also (*Urtica dioica*, *Rumex obtusifolius*), use both (*Plantago major*), or use nitrate only (*Epilobium angustifolium*). Weeds thus vary in physiology as in systematic position.

The nitrate content of plants varies greatly; it can be very large with high supply and slow utilization. High nitrate contents in pasture plants can cause livestock poisoning. The actual toxic agent is nitrite, produced, in some cases at least, by an enzyme in the plant, as reported for *Tribulus* spp. (Rimington & Quin, 1933) and *Salvia reflexa* (Williams & Hines, 1939). Nitrate poisoning is often caused by oats (Bradley, Eppson & Beath, 1940; Whitehead, Olson & Moxon, 1944), whose nitrate content is very variable even between plants of the same variety and from the same plot, but can reach 15·8% (as KNO_3) of the dry weight. A KNO_3 content of 20% has been reported for Johnson grass (*Andropogon sorghum*) (Pease, 1897). Suneson (1932, 1933) found striking differences between species of marine algae in nitrate accumulation. *Laminaria* spp., but no other brown algae, accumulated nitrate; it was present in many red algae but absent from all ten *Polysiphonia* spp. tested.

Nitrogen uptake at different levels of supply. Wadleigh (1944) showed that cotton plants with culture solutions containing 8, 25, 75 and 225 p.p.m. of nitrogen as nitrate took up respectively 87, 85, 88 and 34% of their supply. Hoener & De Turk (1938) found that low- and high-protein strains of corn (*Zea mays*) grown in water culture with 25, 50, 100 and 200 p.p.m. of nitrate had the same amount of nitrogen per plant, except at 100 p.p.m., where the high-protein strain had twice as much as the other.

Reduction of nitrate. Various bacteria form nitrite from nitrate (Woods, 1938; Aubel, 1938; Korsakova, 1941; Lascelles & Still, 1946) and go on to form ammonia, probably via hydroxylamine. The bacterium *Haemophilus influenzae* reduces nitrate to nitrite only if grown with porphyrins containing the vinyl group (Granick & Gilder, 1946). A *Neurospora* mutant is known which can use ammonia or nitrite but not nitrate (Horowitz *et al.* 1945). Mikhlin (1938) and Lemoigne, Monguillon & Desvaux (1937*a, b*) report evidence of nitrite reduction to hydroxylamine in higher plants, the latter authors suggesting ascorbic acid as the reducing agent. They also report combined hydroxylamine in fresh leaves. Interpretation of these results is complicated by the contention of Steinberg (1939) that reports of nitrite and hydroxylamine in plants are due to nitrohydroxylaminic acid ($H_2N_2O_3$). He found nitrate, ammonium and sodium nitrohydroxylaminate good nitrogen sources for *Aspergillus niger*, nitrite and hydroxylamine being unavailable and toxic in moderate concentrations. Hydroxylamine is also toxic to the green alga *Chlorella* (Ludwig, 1938) and nitrite to *Baeria chrysostoma* (Compositae) (Loo, 1946).

Nitrite and hydroxylamine are logical intermediates in nitrate reduction, but both appear toxic and poorly assimilated. Ammonia itself is toxic, but when absorbed is normally used in synthesis of amino-acids or amides. Burström (1945), in a review covering the whole field of nitrate assimilation, suggests that free nitrate is in equilibrium in the plant with nitrate combined with some organic constituent, the reduction process passing the oxidation levels of nitrite and of hydroxylamine with the nitrogenous intermediates in organic combination. At the nitrate oxidation level a shift of equilibrium in the direction of inorganic nitrogen leads to nitrate accumulation; at the nitrite level no similar accumulation occurs, nitrite being removed in side reactions either as free nitrogen (see p. 18) or as ammonia. The puzzling temporary increase in nitrate in dark-cultured leaves of tobacco (Vickery, Pucher, Wakeman & Leavenworth, 1933; Vickery, Pucher, Schoenheimer & Rittenberg, 1939), Swiss chard, tomato (M.C. McKee & Lobb, 1938) and barley (H. S. McKee, 1938) may be interpretable along these lines, though its causes are still quite obscure.

The fate of the oxygen of the nitrate molecule is uncertain. There is evidence (see p. 18) that nitrate utilization favours formation in plants of oxidized compounds. Gilbert & Shive (1945) report that soybean, oat and tomato roots grown with nitrate have a higher respiration rate than those grown with ammonium or without nitrogen, and consider the extra carbon dioxide output directly associated with the oxygen made available in nitrate reduction. The higher respiration rate may, however, be a measure of protein content, known to be highly correlated with respiratory activity; isolated roots of *Pandanus Veitchii* form more protein with nitrate than with ammonium (Sideris, Krauss & Young, 1937). The origin of the extra carbon dioxide is not clear from the data of Gilbert & Shive; detached leaves of barley seedlings grown with nitrate produce in darkness over the first 24 hr. more carbon dioxide than leaves from ammonium plants, but oxygen uptake is correspondingly higher (McKee, 1938).

Nitrate reduction being an endothermic process must eventually be coupled with respiration in tissues where this is the only source of energy. Warburg & Negelein (1920) formulated the results of their work on *Chorella* as:

$$HNO_3 + H_2O + 2C = NH_3 + 2CO_2 + 162{,}000 \text{ cal.},$$

the carbon being at the oxidation level of carbohydrate. The equation assumes an anaerobic process, but in wheat roots Burström (1939*b*) showed that nitrate reduction is associated with aerobic respiration. The extremely acid medium (containing N/10-$NaNO_3$ and N/100-HNO_3) used by Warburg & Negelein also makes the general applicability of their results doubtful. Yamagata (1934) found essentially constant ratios of carbon, hydrogen and oxygen in *Aspergillus oryzae* grown with ammonium or nitrate plus various carbon sources. The number of oxygen atoms associated with an atom of nitrogen is, however, variable, mainly because the nitrogen content is higher with ammonium. The ratio of the number of oxygen atoms per nitrogen atom in the nitrate and ammonium series varies considerably, as shown in the table below, computed from Yamagata's data:

Carbon source	Ratio of oxygen atoms per nitrogen atom in hyphae with nitrate and ammonium
Glucose	1·17
Mannitol	1·15
Glycerol	1·58
Ethanol	1·25

In spite of this variability in nitrogen content, the comparative constancy of the relations of carbon, hydrogen and oxygen to one another allowed excellent agreement between computed and observed values for the respiratory quotient with nitrate and ammonium plus 15 sources of carbon. Oxygen uptake seems therefore limited to that required to maintain the hyphal composition by oxidizing excess of carbon or hydrogen to carbon dioxide and water; the oxygen of nitrate is available equally with free oxygen or that combined in carbon compounds.

Preparations from *Bacillus coli* and *Bacterium pyocyaneum* reduce nitrate to nitrite, and, in the latter species, reduce nitrite further. The two reductions are differently affected by cyanide, so that different enzymes may be involved (Yamagata, 1940). With higher plants attempts to isolate such enzymes have been unsuccessful. Various heat-stable extracts capable of reducing nitrate have been made from plant tissues, but their connexion with the normal process is doubtful. Burström (1945) stresses that in wheat roots and fungal hyphae reduction appears to take place at the surface of the cytoplasm, so that intact structures are required and isolation of the system is unlikely.

Influence of metals on nitrate assimilation. Burström (1939*a*, *b*) has shown that manganese is essential in assimilation of nitrate by isolated wheat roots. Other cations tested, including iron, were inhibitory, due perhaps to displacement of absorbed manganese. When absent, manganese is partly replaceable by iron. Noack & Pirson (1939) and Alberts-Dietert (1941) got similar results with *Chlorella*; Maschmann (1939) also reports replacement of manganese by iron in activating the dipeptidases of anaerobic bacteria. Lundegårdh (1939) showed a close association between respiration of wheat roots, also catalysed by a manganese system located at the surface of the cytoplasm, and nitrate assimilation. He suggests as a working hypothesis that intermediates in glycolysis are used in amination. If Burström's formulation is accepted they might be coupled with nitrate-reduction products at a higher oxidation level than that of ammonia. In the green alga *Ulva lactuca* manganese considerably increases growth with nitrate but not with ammonium (A. Kylin, 1943; H. Kylin, 1943). Arnon (1939) found roots of barley plants grown with nitrate several times as rich in manganese as those grown with ammonium; nitrates accumulate in manganese-deficient plants of oats and *Phalaris minor* in conditions where they are absent from plants adequately supplied with manganese (Leeper, 1941). Vlasyuk (1940*a*, *b*) found that manganese improved growth of sugar-beet plants grown in water culture with either nitrate alone or equal amounts of nitrogen as nitrate and ammonium. Growth with ammonium alone was poor but in some cases improved by manganese, possibly through better assimilation of nitrate produced by bacteria. The lowered ascorbic acid content found (Harmer & Sherman, 1943) in manganese-deficient plants may be an effect of reduced nitrate utilization, as nitrate appears to favour formation of oxidized compounds (see p. 18). Boron-deficient *Tropaeolum majus* shows a progressive decrease in nitrate absorption, compared with normal plants, but accumulations of ammonia and carbohydrate suggest amination may be inhibited, as does the high-protein N/non-protein N ratio observed in plants showing symptoms of excess boron (Briggs, 1939). Potassium seems essential for nitrate reduction, as both carbohydrate and nitrate accumulate in potassium-deficient plants (Nightingale, Schermerhorn & Robbins, 1930).

Photosynthetic assimilation of nitrate. Burström (1943*a*, *b*) has shown that nitrate assimilation in wheat leaves requires light, increases with rising light intensity and is correlated with carbon dioxide fixation. Both sugars and nitrogenous compounds appear primary assimilation products, as leaves in the dark, even if containing much sugar, do not assimilate nitrate. Nitrate-containing leaves absorb more carbon dioxide than can be accounted for as sugar, the difference varying with the nitrate assimilated. The assimilation mechanism clearly differs from that in roots. Protein and carbohydrate appear as alternative products of photosynthesis. Burström (1945) quotes similar conclusions on other grounds published in highly inaccessible papers by Sapozhnikov in 1894 and Krashenninikov in 1901, and also cites in this connexion von Körösy (1913), whose data show in photosynthesizing leaves of *Robinia pseudoacacia* much greater gains in heat of combustion than can be accounted for by increases in sugar, starch and fat. This he attributes, though the point is not definitely established by his data, to an increase in cellulose. A significant formation of complex nitrogenous compounds is unlikely, as nitrate could not be detected in the leaves.

Eisenmenger (1933) showed that nitrate disappears sooner in the light than in the dark from young tobacco plants no longer supplied with it. Barley seedlings grown in light with a high nitrate supply contain much nitrate 8 days after germination, but at 12 days the nitrate content is low and by 16 days none can be detected; in the dark nitrate content per shoot reaches a maximum at 12 days and then decreases steadily, though some is still present at 28 days (McKee, 1938). Petrov (1917) found that seed plants supplied with sugar used ammonia better than nitrate in the dark, the position being reversed in light. Marthaler (1937) reports, however, a relatively greater uptake of nitrate in darkness than in light by *Rumex obtusifolius*. The observation (White, 1937) that the optimum nitrate supply for *Lemna* increases with light intensity is in accordance with Burström's data. The relative importance of roots and leaves in nitrate assimilation is hard to assess. The nitrate content of leaves may vary from very high, as in examples quoted above, to zero; the latter may imply merely that utilization equals intake. Sideris, Young & Chun (1947) found nitrate in pineapple leaves only with high nitrate supply. Buckwheat (*Fagopyrum esculentum*) and *Bryophyllum calycinum* with adequate but not excessively high nitrate supply show appreciable amounts of nitrate in the leaves. The main accumulation is in the stem, possibly because it is not an organ of active metabolism (Pucher, Wakeman & Vickery, 1939; Pucher, Leavenworth, Ginter & Vickery, 1947*a*, *b*). The pineapple (Nightingale, 1942*a*) also stores nitrate in the stem. Entry of nitrate to leaves may vary during their ontogeny. There is evidence (see p. 41) that capacity for protein synthesis decreases with age; if the nitrate intake remains constant this should cause it to accumulate in older leaves. Young, but not mature, citrus fruits take up nitrogen supplied to the tree (Finch, 1944; Jones, Bitters & Finch, 1944).

Reduction of yield by low nitrogen supply is less for potato and other root crops than for crops where leaves or pods are harvested (Ware & Johnson, 1944).

(*b*) *Relations between assimilation of nitrogen and of phosphorus*

There is evidence from several sources of interactions, probably indirect, between the uptake of nitrogen and of phosphorus. Absorption of phosphate by the marine diatom *Nitzschia closterium* increases with the nitrate content of the medium, but nitrate absorption is independent of the phosphate content (Ketchum, 1939). In field experiments with pineapple in Hawaii, on the other hand, Nightingale (1942*b*) found phosphate absorption, from soils in which it is rather deficient, impeded by increased nitrate supply. Young plants of *Phalaris tuberosa* absorb both nitrogen and phosphorus rapidly, but absorption of nitrogen is concentrated much more into the early stages of growth than that of phosphorus (Richardson, Trumble & Shapter, 1932). Burström (1947) states that cell multiplication in isolated roots varies with phosphate supply, but cell elongation is scarcely affected; the position is reversed with nitrate. This is in agreement with observations by Avery & Pottorf (1945*a*) that in kohlrabi (*Brassica caulorapa*) auxin content increases with nitrate supply. Diploid cabbage seedlings, however, are similar in nitrogen content to tetraploids but have 2–3 times as much auxin (Avery & Pottorf, 1945*b*). In the coleoptile of *Zea mays* the protein content of cells increases 9·5 times during elongation (Frey-Wyssling & Blank, 1940), suggesting a further reason for the dependence of this process on nitrogen supply.

(*c*) *Organic nitrogen*

Urea is probably the organic compound most studied as a source of nitrogen for plants. Its use goes back to Hampe (1865), but the earlier experiments did not exclude bacterial transformation before entry into the plant. Hansteen (1899) studied the uptake of urea in sterile conditions, and it has since been shown (Yamaguchi, 1930; Tanaka, 1931), using microchemical tests with the Fosse-Klein xanthydrol reagent, to enter the plant unchanged. Species used were *Sisyrinchium bermudiana*, *Plantago major* and *Brassica chinensis*. Pirschle (1929) found higher urea-splitting activity in roots of fourteen species grown with urea than in controls with ammonium nitrate. Urea is reported a better nitrogen source than ammonium for *Baeria chrysostoma* (Compositae) (Loo, 1946), tobacco (Beaumont, Larsinos, Piekenbrock & Nelson, 1931), citrus (Hilgeman, 1941) and equal for peach trees (Nasharty, 1944).

Observations on the utilization by plants of nitrogen in other organic compounds are scattered through the literature. Barley is stated to grow with lysine as its only nitrogen source (Virtanen, Laine & von Hausen, 1936) and uninoculated peas to use the nitrogen of aspartic and glutamic acids in preference to inorganic nitrogen (Virtanen, 1938, 1939; Virtanen & Linkola, 1946). Glutamic acid and alanine alone of a series of amino-acids (including aspartic acid and glycine) showed no inhibition of root growth in cress seedlings when supplied at 1000 p.p.m. in sterile culture (Audus & Quastel, 1947). White (1939), however, found alanine toxic at 3 p.p.m. to isolated tomato roots and glycine satisfactory as sole nitrogen source. The nutrient requirements of roots seem to vary greatly with species, e.g. isolated roots of *Trifolium pratense* grow much better with yeast extract than with glycine, and the position is reversed for *Helianthus annuus* (White, 1939). Tobacco seedlings supplied aseptically with combinations of amino-acids grew better than with any one amino-acid tested (Pratesi & Ciferri, 1946). Knudson (1933) tried several amino-acids as nitrogen sources for orchids but none was utilized. Leaves of the insectivorous *Drosera capensis* supplied with glycine, urea, asparagine, peptone, gelatine or insect bodies accumulate soluble nitrogen, translocated later to other parts of the plant (Oudman, 1936).

Nitrogen requirements of fungi vary greatly in specificity. Some, e.g. *Aspergillus niger*, can use nitrate; others require complex nitrogenous compounds. The wood-destroying fungi *Polyporus betulinus*, *Fomes pinicola* and *Polystictus versicolor* can use inorganic nitrogen, though not urea, but do much better with aspartic acid, glutamic acid, peptone or protein (La Fuze, 1937). The ringworm fungus *Trichophyton mentagrophytes* cannot use ammonia but grows on any of fourteen amino-acids tested; no mixture of these is as effective as protein hydrolysates (Robbins & Ma, 1945). *Nematospora gossypii* grows on protein hydrolysates, but attempts to use synthetic mixtures of amino-acids were unsuccessful (Farries & Bell, 1930). Aspartic acid has a special place in its nitrogen requirements (Buston, Kasinathan & Wylie, 1938).

(*d*) *Nitrogen fixation*

A vast number of compounds shows the reactivity of combined nitrogen, but only a few organisms can assimilate its very unreactive diatomic molecules. Nitrogen organically combined by living organisms is nevertheless derived ultimately from elemental nitrogen in the atmosphere, mainly through the activities of nitrogen-fixing species. These play an essential part in the nitrogen cycle as now found in nature, but may not always have done so, as Oparin (1938), largely on astrophysical grounds, argues that organisms first developed on earth in an atmosphere composed essentially of methane and ammonia. Small amounts of nitrogen become available to organisms through combination with oxygen by electrical discharges in the atmosphere and through emission of ammonia—probably formed by steam acting on metallic nitrides—from volcanoes. Appreciable amounts of nitrogen compounds, mostly ammonia, are carried down in rain (Wilson, 1921), but probably arise by burning of fuels containing combined nitrogen and by decomposition of organic matter.

The agricultural use of 'artificial' nitrogenous fertilizers has in recent times made available in another way the nitrogen of the atmosphere to plants and so, indirectly, to animals. It should be realized, however, that not all 'artificial' nitrogenous fertilizers are products of industrial fixation processes; an organic origin is probable for nitrate got by mining and certain for ammonia derived from coal. Allowing for all these subsidiary sources of combined nitrogen, it is still true that nitrogen fixation by micro-organisms is the dominant means making nitrogen from the atmosphere available for other forms of life. To draw up a balance sheet for the transformations of nitrogen is a task of obvious difficulty; it has, nevertheless, been attempted for the United States by Lipman & Conybeare (1936). They estimate yearly losses of nitrogen from the land by leaching, erosion and sewage disposal to be considerably greater than replacements, the order of importance of replacement sources being: symbiotic bacteria, free-living bacteria, nitrogenous compounds carried down in rain, manure, artificial fertilizers, the last providing about one-fifth as much nitrogen as manure and one-tenth as much as symbiotic bacteria.

Their great importance in the economy of nature, and the smoothness with which they carry out a process whose industrial equivalent requires extreme conditions, have long made nitrogen-fixing micro-organisms the object of intensive research. Their metabolism and its agricultural implications have both been studied in many countries, but in the former field the schools of Wilson in Madison, U.S.A., and Virtanen in Helsinki, Finland, have been outstanding in recent work. The field has been reviewed by representatives of each school (Wilson & Burris, 1947; Virtanen, 1947); Wilson (1940) has also published an excellent monograph covering the whole development of the subject, with a particularly good account of the nineteenth-century work on which our present knowledge is based.

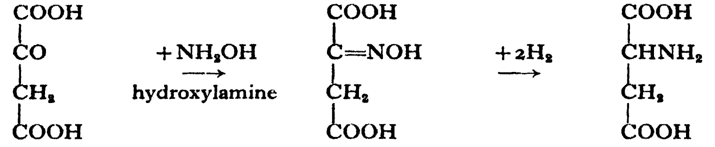
Fixation of nitrogen, although reported for a number of other micro-organisms, has been extensively studied only with the symbiotic bacteria of the root nodules of leguminous plants and with the free-living *Azotobacter*. The symbiotic system is particularly difficult to study, as both organisms seem essential for fixation, which has not been demonstrated for either partner alone. Frequent reports in the early literature of nitrogen fixation by free-living legume bacteria have since been refuted in more critical experiments (Löhnis, 1930; Burris, Eppling, Wahlin & Wilson, 1943). The latter authors, using the N^{15} isotope, provided unequivocal evidence of fixation with *Azotobacter vinelandii*, *Clostridium pasteurianum* and the blue-green alga *Nostoc muscorum*, but detected none with free-living legume bacteria, germinating peas or cell-free *Azotobacter* extracts. A claim by Bach, Yermoleva & Stepanian (1934) that such extracts could fix nitrogen aroused considerable interest but has not been confirmed by other workers (Roberg, 1936; Allison, Hoover & Minor, 1942). Studies on the symbiotic system have therefore used inoculated legume plants, often grown by elaborate methods to avoid contamination with free-living nitrogen-fixing bacteria. Nightingale (1937*a*) has stressed that conditions in such cultures are far from normal, owing to the effects on aeration and light intensity, but these objections are largely met by Virtanen's method of growing the roots aseptically and the tops exposed to air. Excised legume nodules, which might be thought more convenient than intact plants, have given too erratic results (Virtanen & Laine, 1937*b*; Allison, Hoover & Minor, 1942; Burris *et al.* 1943; Wilson & Burris, 1947) to be promising experimental material. Excised nodulated pea roots, however, show more consistent fixation (Burris *et al.* 1943).

Excretion from nodules. Excretion of nitrogenous compounds from legume nodules, and their uptake by associated non-legumes, have been widely studied. Excretion has been regularly and consistently observed by the Finnish workers, but elsewhere erratically or not at all. Wilson (Wilson & Burton, 1938), though able at Helsinki to confirm Virtanen's positive results, could not reproduce them reliably at Madison. He concludes that excretion requires a rather critical balance, sensitive to climatic conditions, of carbohydrate and nitrogen in the legume and found, as did Strong & Trumble (1939), that shading legumes induced excretion in some cases. It seems possible the very long cool days in the growing season at Helsinki may have some connexion with the regularity with which excretion is observed there. Chailakhyan & Megrbyan (1944) report that long-day conditions increase nodule formation in legumes, probably through higher production of carbohydrate and of growth-hormone. Virtanen (1947) also stresses that the physical properties of the medium in which the plants are grown may determine whether excretion takes place.

Demidenko & Timofeyeva (1937*a*, *b*) reported a transfer of nitrogen from peas to oats, and also uptake by oats of ammonia excreted by *Azotobacter*; it is stated to require the root excretions of a 'suitable' plant, thus taking part in a loose symbiotic association. Lebedev (1940) reported a significant transfer of nitrogen from lupins to hemp growing in association with them, and Nowotnówna (1937) got similar results with several mixed cultures. Transfer to associated plants of so much of the nitrogen fixed by legumes that they show signs of nitrogen shortage has been reported from Helsinki (Virtanen, von Hausen & Laine, 1937*a*, *b*; Virtanen & Laine, 1939), but rarely elsewhere. Essentially negative results have been reported by many workers, e.g. Bond (1938, 1941), Bond & Boyes (1939), Chapman (1943), Engel & Roberg (1938), Ludwig & Allison (1937), Romashev (1939), Shapter (1939), Swaby (1945) and Trumble & Strong (1937). Transfer of nitrogen from legumes to associated plants thus seems more a reaction to rather unusual conditions than a normal physiological process. The nitrogen fixed by legumes probably becomes available to other plants normally by decomposition of dead residues. Jensen & Frith (1944) found that nitrogen in the root nodules of lucerne and subterranean clover was converted to nitrate in the soil much more rapidly than that of the roots or tops. Trumble & Shapter (1937) report that 8 weeks after subterranean clover grown in association with *Phalaris tuberosa* was harvested, about 30% of the nitrogen in its roots had been transferred to the grass. The surprising report by Leonard & Reed (1930) that the non-nodulated legumes *Cassia occidentalis* and *C. tora* are as effective for green manuring as several species of nodulated legumes may indicate merely that in their conditions the benefit of green manuring is due to factors other than the nitrogen added to the soil.

Mechanism of fixation. Virtanen's school has based far-reaching arguments on the mechanism of fixation on the nature of the products observed in excretion. Virtanen & Laine (1937*a*) demonstrated excretion of amino-nitrogen from the root nodules rather than the roots by growing peas in sterile culture in a flask with an inner tube within which the sand was inoculated with nodule bacteria. Nodulation and amino-acid excretion occurred in the inner tube but not in the uninoculated outer part of the flask. Excretion begins as soon as the nodules are formed and is at a maximum before flowering (Virtanen *et al.* 1937*a*).

Aspartic acid is the main nitrogenous compound excreted, but β-alanine and oximinosuccinic acid are also found (Virtanen *et al.* 1936; Virtanen & Laine, 1937*c*, 1939). The β-alanine is regarded as a secondary product formed by legume bacteria decarboxylating aspartic acid (Virtanen, Rintala & Laine, 1938). The presence, in small amounts, of oximinosuccinic acid is considered (Virtanen, 1936) to show hydroxylamine as the first product of nitrogen fixation, combining with oxalacetic acid to form aspartic acid as shown below:



$$\begin{array}{l} \mathrm{COOH} \\ | \\ \mathrm{CO} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{COOH} \end{array} \quad \xrightarrow[\text{hydroxylamine}]{+\mathrm{NH_2OH}} \quad \begin{array}{l} \mathrm{COOH} \\ | \\ \mathrm{C{=}NOH} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{COOH} \end{array} \quad \xrightarrow{+2\mathrm{H_2}} \quad \begin{array}{l} \mathrm{COOH} \\ | \\ \mathrm{CHNH_2} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{COOH} \end{array}$$

Oxalacetic acid Oxaminosuccinic acid Aspartic acid

Substances containing the oxime (CNOH) group have also been found in the culture medium of *Azotobacter chroococcum* and *A. beijerinckii* fixing nitrogen or supplied with nitrate or nitrite (Endres, 1936; Virtanen, 1939). Formation of aspartic acid from fumaric acid and ammonia is considered less likely, as aspartase is lacking from nodules and nodule bacteria (Virtanen, 1936). Suomalainen (1940) claims to have isolated amino-oxalacetic acid from roots of inoculated peas, and suggests it is an intermediate in the formation of aspartic acid. Oxalacetic acid itself has been estimated in pea and red clover plants by several methods (Virtanen & Laine, 1938; Virtanen, Laine & Roine, 1938; Virtanen & Arhimo, 1939*a*, *b*; Virtanen, Arhimo, Sundmann & Jännes, 1943). Wyss, Burris & Wilson (1939) could not confirm its presence, but it seems likely, in view of its metabolic relationships (see p. 22), to occur in plant tissues even if not accumulating in readily measurable amounts. Biotin, required for rapid growth by most strains of legume bacteria tested (Wilson & Wilson, 1942), is involved in the formation of oxalacetate from pyruvate in other bacteria (Shive & Rogers, 1947; Lardy, Potter & Elvehjem, 1947). There is, however, no rigorous evidence that either oxalacetic acid or hydroxylamine plays the central role attributed to it in nitrogen fixation. More recent papers from Virtanen's laboratory lay less stress on oxalacetic acid, treating oxime formation as a side reaction, hydroxylamine being mainly reduced to ammonia, which forms glutamic acid (see p. 25) with α-ketoglutaric acid (shown to be present in legumes) (Virtanen, 1947; Virtanen *et al.* 1943). Glutamic acid, originally reported absent from root excretions of nodulated legumes, has now been found in them (Virtanen, Linkola, Hakala & Rautanen, 1946).

The work on excretion is of great intrinsic interest, and may well indicate the type of compound in which nitrogen is transported from the nodule to the legume plant, especially as uninoculated peas and clover grow well with aspartic or glutamic acid and use them in preference to inorganic nitrogen sources when both are supplied (Virtanen, 1938, 1939; Virtanen & Linkola, 1946), but seems incapable of providing definite evidence on the mechanism of fixation. The compounds excreted may well be formed as a result of nitrogen fixation, but their nature is not specific evidence for any particular fixation mechanism.

Wilson and his co-workers have long held ammonia the most likely initial product of fixation, and now (Wilson & Burris, 1947) consider the evidence in its favour decisive. Most of their critical evidence (Burris, 1942; Burris & Wilson, 1946*a*) is from experiments with *Azotobacter* using the stable nitrogen isotope N^{15}, which has replaced the gasometric method of Hurwitz & Wilson (1940)—itself a considerable advance on the Kjeldahl method—as the most precise and sensitive method available. *Azotobacter* supplied with free nitrogen or ammonium salts containing N^{15} accumulated the heavy isotope in glutamic and aspartic acids first and later in other nitrogen fractions. This distribution is also found in rats fed N^{15} in ammonium citrate or amino-acids (Foster, Schoenheimer & Rittenberg, 1939; Rittenberg, Schoenheimer & Keston, 1939) and in tobacco plants given N^{15} in ammonium chloride (Vickery, Pucher, Schoenheimer & Rittenberg, 1940). The accumulation in glutamic acid appears to be primary, and that in aspartic acid probably produced by transamination, which occurs readily in *Azotobacter* (Lichstein & Cohen, 1945).

Using *A. vinelandii*, Burris & Wilson (1944) demonstrated uptake of nitrogen from ammonia within 1 min., though 30 min. was required with nitrate. Ammonia and its precursors inhibit fixation in *A. vinelandii*, but nitrite and nitrate do so only after a period of adaptation, suggesting they are first converted to ammonia (Wilson, Hull & Burris, 1943). This conclusion was reached also by Aso, Migita & Ihda (1939) from studies of the rates of uptake of nitrate, nitrite and ammonia by *A. chroococcum*, *A. beijerinckii* and *A. agile*, and is supported by the results of Horner & Allison (1944). Many workers, e.g. Winogradsky (1939), have found ammonia in the medium in which *Azotobacter* has been grown, but Burk & Horner (1936) held it a catabolic product rather than an intermediate in fixation. Complete refutation of this view is difficult, but it is at least not supported by the observation of Butkevich & Kolesnikova (1941) that ammonia accumulates most in young cultures well supplied with carbohydrate and actively fixing nitrogen.

The general biochemical relationships of the fixation process have been extensively studied by Wilson's school. They have shown carbon monoxide in very low concentrations is a non-competitive inhibitor of fixation in legumes, *Azotobacter* and *Nostoc muscorum*, and in higher concentration inhibits the uptake of combined nitrogen also (Lind & Wilson, 1941, 1943; Ebersole, Guttentag & Wilson, 1943; Wilson & Burris, 1944). Hydrogen is a specific, competitive inhibitor in *Azotobacter* (Wyss & Wilson, 1941), nodulated legumes (Wilson & Umbreit, 1937) and *Nostoc* (Burris & Wilson, 1945, 1946*b*). These results all suggest the essential similarity of fixation in the legumes, *Nostoc* and *Azotobacter*. The latter, however, has a powerful hydrogenase, apparently associated with fixation (Lee, Wilson & Wilson, 1942) but not found in legume bacteria (Wilson & Wilson, 1943) or nodules (Wilson, Burris & Coffee, 1943).

Studies of the distribution of nodule nitrogen among various groups of compounds show that though the total nitrogen concentration is higher than in the rest of the plant, the same types of compounds are present (Orcutt, 1937; Umbreit & Burris, 1938).

A theoretical analysis of possible mechanisms of fixation is given by Wilson & Burris (1947).

Legume root nodules contain a red pigment identical with or closely related to animal haemoglobin (Kubo, 1939; Keilin & Wang, 1945; Common, 1945) and truly reversibly oxidizable (Keilin & Smith, 1947). It may be directly involved in the fixation process (Virtanen, 1947), but seems more likely to be an oxygen carrier, for there is physiological (Pietz, 1938; Allison, Ludwig, Hoover & Minor, 1939, 1940) and anatomical (Frazer, 1942) evidence of limited oxygen supply inside the nodules, and the bacteria are aerobic. The effects of carbon monoxide on the fixation process in legumes and on animal respiration may thus be analogous (Keilin & Wang, 1945; Keilin & Smith, 1947), but this view fails to explain how carbon monoxide affects fixation in *Azotobacter* and *Nostoc*, which contain no haemoprotein and are less likely than the legume bacteria to live in conditions of partial anaerobiosis. It should also be remembered that several nitrogen fixers in the genus *Clostridium* are anaerobic, in contrast to the strict aerobe *Azotobacter*.

Effects of metals on fixation. Much attention has been given to the effect of molybdenum on nitrogen-fixing organisms since the pioneer papers of Bortels (1930, 1936, 1940) claimed it essential for fixation by *Azotobacter*—as was confirmed by Krzemieniewski & Kovats (1936) and Matuashvili (1947)—and the blue-green algae *Anabaena* and *Nostoc*. Jensen & Spencer (1947) found that nitrogen fixation by *Clostridium butyricum* and *Cl. acetobutylicum* responded markedly to molybdenum supply. Legume seeds contain molybdenum in higher concentrations than those of most other plants (Bertrand, 1939; Vinogradova, 1943); root nodules from legumes grown in a sand low in molybdenum had 5–15 times the molybdenum content of the roots, which had in turn more than the tops (Jensen & Betty, 1943). There is, however, evidence also that molybdenum is required by plants which do not fix nitrogen. Culture experiments have shown its essentiality for tomato (Arnon & Stout, 1939), oats (Piper, 1940), *Lemna* (Steinberg, 1941), and *Aspergillus niger* (Steinberg, 1936, 1937), and make it probable for asparagus and lettuce (Arnon, 1938). It is a constant constituent of the seeds of *Taraxacum kok-saghyz* (Compositae) (Borovik, Bergman & Borovik-Romanova, 1943) and of several Cruciferae, the latter having as much as legume seeds (Bertrand, 1939). With *Aspergillus niger*, Steinberg (1937, 1939) showed that the effect of molybdenum deficiency is increased by supplying nitrogen as nitrate, compared with ammonia or assimilable organic nitrogen compounds, and suggested that molybdenum is concerned in the reduction of nitrate to ammonia. Horner, Burk, Allison & Sherman (1942) found that, though eight strains of *Azotobacter chroococcum* and one of *A. vinelandii* showed ten- to thirty-fold increases in nitrogen fixation with added molybdenum, two of *A. agile* and three of *A. vinelandii* gave only two-fold increases owing to higher fixation without it, and some strains could not be shown to require it at all. Molybdenum may thus be concerned with some phase, other than fixation, of the nitrogen metabolism of nitrogen-fixing organisms.

More definite evidence that molybdenum is essential for the specific process of nitrogen fixation is given by Jensen (1945), who found in molybdenum-deficient lucerne plants large but inefficient nodules fixing per unit weight only 40% as much nitrogen as normal nodules. Lucerne plants supplied with combined nitrogen took up less molybdenum than those fixing free nitrogen, as observed also by Jensen & Betty (1943). Field responses to molybdenum by legumes have been observed by Dmitriev (1939*a*, *b*), Anderson (1946) and Anderson & Thomas (1946). The Australian workers found that legumes responded to molybdenum in conditions where grasses did not; legumes without added molybdenum, though better nodulated than those with it, fixed little nitrogen. Supplied with ammonium salts or nitrates they gave very little response to molybdenum. Trumble & Ferres (1946) also got responses to molybdenum by five species of inoculated legumes in pot and field trials. Bortels (1936) found that vanadium had effects with *Azotobacter* similar to, but less marked than, those of molybdenum. This was confirmed by Horner *et al.* (1942), and extended to *Clostridium* by Jensen & Spencer (1947), but Jensen & Betty (1943) and Anderson & Oertel (1946) with lucerne and Dmitriev (1939*a*, *b*) with red clover found vanadium ineffective. Iron is also essential for *Azotobacter* (Krzemieniewski & Kovats, 1936; Horner *et al.* 1942) and boron for nodulation of legumes (Brenchley & Thornton, 1925). Responses to boron by nodulated legumes in field experiments are reported by Dmitriev (1939*a*, *b*) and Lebedev (1940). Matuashvili (1947) states that *Azotobacter* does not require boron for fixation.

Integration of metabolism in host and nodule. Ali-Zade (1941) has published some remarkable data on protein synthesis and hydrolysis *in vitro* by preparations of legume nodules. Synthesis predominated in brei from nodules of plants at the stage of flower-bud formation, and hydrolysis when the plants were at the flowering and, especially, at the fruiting stage. Nodule preparations from regularly disbudded plants showed considerable synthesis at a time when those from comparable plants allowed to flower and set seed gave considerable hydrolysis, which could be reduced by adding the juice of leaves from disbudded plants. Brei from the nodules of flowering plants gave slight hydrolysis alone, but with juice from the leaves of plants forming buds gave synthesis, juice from the leaves of fruiting plants markedly increasing the hydrolysis. The data suggest that protein metabolism in the nodules is closely integrated with that of the host plant, and is controlled by the latter through a hormone mechanism.

Fixation in species other than legumes and Azotobacter. Many organisms, besides the much-studied legume bacteria and *Azotobacter*, have been reported to fix free nitrogen. With some, e.g. *Nostoc muscorum* and some *Clostridium* species, the evidence seems decisive, but the authenticity of the reports is often open to doubt, usually through lack of further study after publication of the initial data.

Fritsch & De (1938) have suggested that the growing of rice in India in the same fields for many years without manuring is made possible by the extensive growth of nitrogen-fixing blue-green algae in the waterlogged soil. Chaudhuri (1940) considers, however, that the algae are not themselves important fixers of nitrogen, but that their dead remains supply nutriment for nitrogen-fixing bacteria. Allison, Hoover & Morris (1937) state that only a few species of blue-green algae fix nitrogen, but that these may be important in the maintenance of soil fertility.

Rotmistrov (1941) reports considerable nitrogen fixation by the anaerobic cellulose bacterium *Granulobacter pectinovorum*. Ruben, Hassid & Kamen (1940*a*), using a radioactive nitrogen isotope, claimed a fixation of nitrogen by green barley tissues. This was not confirmed by Burris (1941) using the N^{15} isotope, which is better suited to tracer work than the radioactive one, whose half-life is only 10·5 min.

Nodules believed to be inhabited by nitrogen-fixing organisms have been reported from the roots of many non-legume species, spread through a large number of families. Nogtev (1939) isolated from root nodules of the foxtail grass (*Alopecurus pratensis*) a bacillus which he named *B. alopecuri* and showed to flourish in nitrogen-free media, though fixation was not definitely established. Spratt (1912*b*) isolated from the root nodules of *Podocarpus* (Coniferae) and the related genera *Microcachrys*, *Saxegothaea* and *Phyllocladus* bacteria fixing nitrogen vigorously in culture, as confirmed for *Podocarpus spinulosa* and *P. elata* by McLuckie (1923*a*). Fixation of nitrogen in culture is reported for the bacteria of most of the non-leguminous nodules studied, suggesting that species other than those associated with the legumes are involved, though, in the absence of work by recent critical methods, some reports may perhaps be based on faulty technique, as were those of fixation by isolated legume bacteria. Phillips (1932) found that seedlings of two *Podocarpus* species grew in sterilized soil only if inoculated with nodule bacteria. Spratt (1912*a*) isolated from root nodules of species of *Elaeagnus* and *Alnus* bacteria fixing nitrogen actively in culture. Burrill & Hansen (1917) showed that these nodules did not, as claimed by earlier workers, contain legume bacteria, but left open the possibility of other nitrogen fixers being present. From the root nodules of *Alnus*, von Plotho (1941) isolated *Actinomyces alni* and showed it to fix nitrogen. Symbiotic fixation in this species was demonstrated also by Roberg (1934) and Virtanen & Saastamoinen (1936). Bottomley (1909, 1912) showed that root nodules of *Myrica gale* (Myricaceae) contained bacteria fixing nitrogen in culture; seedlings grew in sterilized nitrogen-free cultures only if inoculated. He found nitrogen-fixing bacteria living in a slimy covering outside the cortical cells of *Cycas* roots. McLuckie (1923*b*) isolated from the large coralloid root nodules of *Casuarina cunninghamiana* bacteria fixing nitrogen vigorously in culture if supplied with carbohydrate, and Mowry (1933) showed by studies of nodulated and non-nodulated plants of the same species and of *C. equisetifolia*, *C. glauca* and *C. lepidophloia* in sand cultures with controlled nitrogen supply that the plants received nitrogen from the nodules. McLuckie (1922*b*, 1923*c*) isolated nitrogen-fixing bacteria from root tubercles of *Macrozamia spiralis* (Cycadaceae) and from rhizomes of *Gastrodia sesamoides*, a non-chlorophyllous saprophytic orchid with a well-developed mycorrhizal system. A similar orchid, *Dipodium punctatum*, had mycorrhiza from which it appeared to get protein by digesting intracellular hyphae (McLuckie, 1922*a*) but no nitrogen-fixing bacteria. Sabet (1946) found that *Fagonia arabica*, *Tribulus alatus* and four *Zygophyllum* species (all Zygophyllaceae) had root nodules with nitrogen-fixing bacteria, and would not grow in sterilized soil unless inoculated. The floating fern *Azolla carolinana* lives symbiotically with a nitrogen-fixing *Anabaena* species (Bortels, 1940).

A rather different type of symbiosis between nitrogen-fixing bacteria and higher plants, described in several early papers, seems not to have been further studied. Several tropical species of Rubiaceae (*Pavetta indica*, *P. angustifolia*, *P. lanceolata*, *P. zimmermanniana* and *Psychotria bacteriophila*) and Myrsinaceae (*Ardisia crispa*) have bacterial nodules in their leaves. Bacteria isolated from *Pavetta zimmermanniana* fix nitrogen vigorously in culture, and occur not only in the leaf nodules but also at growing points and invariably in the seed, each plant being automatically inoculated. Seeds freed from bacteria by a hot-water treatment germinated but grew slowly, and without added nitrogen showed symptoms of nitrogen starvation, though plants from untreated seeds grew well and made appreciable gains of nitrogen. Von Faber (1912), to whom the data are due, concluded 'die mit Bakterien in den Blättern versehenen Rubiaceen den elementaren Stickstoff aus der Atmosphäre sich anzueignen imstande sind'. Similar experiments with *Ardisia crispa* led to the conclusion 'die Entfernung der Bakterien eine vollkommene Hemmung der Entwicklung bedingt'. The species is thus more dependent on bacterial symbiosis than are the legumes, though, unlike them, it responds to nitrogen fertilizers (Miehe, 1916). Rao (1923) confirmed the observations on *Pavetta indica* and got very similar results with *Chomelia asiatica* (Rubiaceae).

Rayner (1922) claimed that mycorrhizal fungi associated with species of the Ericaceae fix nitrogen, though the evidence quoted is not entirely convincing. Duggar & Davis (1916) found that *Phoma betae* fixed much nitrogen in culture, but got negative results with a number of other species of fungi.

The leguminous genera, *Cassia*, *Cercis*, *Ceratonia*, *Gleditschia* and *Gymnocladus* contain species not known to form nodules. Leonard (1939) has shown that nitrogen-fixing bacteria are absent from the roots of *Gleditschia triacanthos*. Thornton (1939) points out that *Gleditschia* belongs to a small group of legumes known from Cretaceous and early Tertiary strata, and suggests that the association between legumes and nodule bacteria began later than that time.

Ecological relations between legumes and other plants in mixed pastures have been studied by Blackman (1938), Blackman & Templeman (1938, 1940) and Trumble & Shapter (1937). The interactions are complex, but with rising external nitrogen supply clovers tend to disappear, probably through shading by vigorous grass growth rather than from direct injury. The effect of symbiotic nitrogen fixation on ecological relations in natural conditions has received little attention. Raup (1941) stresses the slowness of bacterial action and the low level of soil nitrate in arctic and subarctic conditions, and suggests that plants in these areas must either be adapted to a low nitrogen intake or have a means of utilizing atmospheric nitrogen. Species of *Alnus* and *Shepherdia* (Elaeagnaceae) are prominent in such regions in Canada, and are probably nitrogen fixers; legumes seem poorly represented. In more extreme arctic conditions (Greenland), lack of carbohydrate due to the very short growing season appears more important than nitrogen shortage (Wager, 1938). Legumes are absent from the area studied. In Australia many coastal areas of highly leached, very sandy soils develop a characteristic heath flora. Species of Leguminosae and of *Casuarina* are prominent in this association, but the dominant families are Myrtaceae and Proteaceae, with Rutaceae often as prominent as Leguminosae. The nitrogen relations of plants growing naturally in conditions of low nitrogen supply offer an almost untouched field for investigation. Schanderl (1943) claims that in such situations plants of many non-leguminous families fix gaseous nitrogen. The cumulative evidence against this conclusion is so great that it can hardly be accepted without independent confirmation of the results by a critical method.

LOSSES OF NITROGEN FROM PLANTS

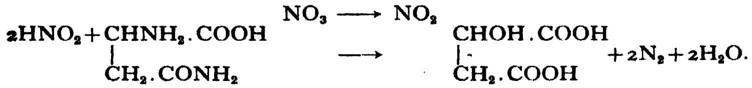
General. Plants, unlike animals, show considerable economy in using nitrogen. Losses occur in various ways, but its excretion is not a constant feature of plant metabolism. Some nitrogen is lost in organs regularly shed—leaves, bark, small branches and the non-seed parts of fruits. Such losses, however, are minimized by its transfer from senescent leaves to perennating parts of the plant or to seeds. A significant part of the total nitrogen of a plant may be laid down in developing seeds, but this of course represents transfer to the next generation, not absolute loss. In tobacco plants the proportion of the total nitrogen in the seed-pods shows a steep linear increase from about 2 to 33 % between the ages of 60 and 120 days, and the form of the curve suggests further increase later (Vickery, Pucher, Leavenworth & Wakeman, 1935). No similar figures for perennials seem available, but a very rough calculation indicates that an apricot tree uses between 50 and 100 g. of nitrogen annually in seed formation; the figure for leguminous trees or for other species, e.g. *Bertholletia excelsa*, *Artocarpus integrifolius*, producing large seeds in quantity, might well be greater. In *Hevea*, however, seed production increases with phosphorus but not nitrogen supply (Haines, 1946).

Loss through roots. Pryanishnikov and his co-workers (see, for instance, Pryanishnikov, 1928) observed excretion of ammonia from carbohydrate-deficient plants, especially in acid media. This has been confirmed (Arenz, 1941, 1942*a*, *b*) with rye, barley, sunflower, pea, broad bean, lupin and clover seedlings. The legumes had a greater capacity for storing ammonium, but here, too, its excretion by the roots could be induced by reduction of carbohydrate reserves. Ivanov & Krupkina (1929) showed excretion by four yeast species of nitrogenous substances into the medium during fermentation.

Comparatively large organic molecules can also leave the plant by the roots. Wheat and pea roots exude sugar and adenylic acid nucleotides, and wheat flavones also (Lundegårdh & Stenlid, 1944). Sugar is also exuded through petiolar hairs of *Impatiens sultani* (Groner, 1939); exudation of glutamine from leaves is discussed on p. 32.

Harmful root excretions have been postulated to explain the incompatibility of some species when grown together. The phenomenon seems well established, *Poa pratensis* especially being sensitive to the presence of certain other species (Trumble & Davies, 1934; Ahlgren & Aamodt, 1939), but Loehwing (1937) shows explanations other than excreted toxins to be possible.

Losses as free nitrogen. Another mode of nitrogen loss is associated with the presence of nitrite, and may, as suggested by Mothes (1938) and Burström (1945), provide a means of avoiding its accumulation in toxic concentrations. The reaction seems first to have been observed in plants by Irving & Hankinson (1908), who showed that *Elodea*, *Vallisneria*, *Potamogeton*, *Iris*, *Vicia faba* and various grasses reduce nitrate to nitrite and give off gaseous nitrogen. They suggested as a possible mechanism:



$$2HNO_2+\begin{array}{l}CHNH_2.COOH\\ |\\ CH_2.CONH_2\end{array}\xrightarrow{NO_3 \longrightarrow NO_2}\begin{array}{l}CHOH.COOH\\ |\\ CH_2.COOH\end{array}+2N_2+2H_2O.$$

Nitrite, in acid media, liberates nitrogen from amino-compounds in general. Pearsall & Billimoria (1937) found considerable losses of nitrogen from the green alga *Chlorella vulgaris* grown with nitrate in the dark. They also found losses from leaves of *Narcissus pseudo-narcissus* floated on nutrient solutions containing nitrate or ammonium salts of organic acids, but, unlike Irving & Hankinson (1908), none with asparagine. Fischer (1936) states that leaves held with their petioles in water lose much nitrogen by diffusion into the water. His experimental technique is criticized by Chibnall (1939) and his results disagree with those of various other workers, e.g. Mothes (1938), who found insignificant nitrogen loss from leaves of *Phaseolus*, *Nicotiana*, *Hippeastrum aulicum* and *Agapanthus umbellatus* held in water. In press-juice of leaves and in fungal cultures large losses of nitrogen occurred on addition of nitrite, but not with nitrate or ammonium. Pennington (1942) gives evidence for appreciable nitrogen loss, probably mainly by bacterial action, from lake waters. Vickery, Pucher, Wakeman & Leavenworth (1946) found considerable losses of nitrogen, greater in the dark than in light, from plants of *Narcissus poeticus* developing from bulbs in distilled water. They suggest that the nitrite-amino reaction may be involved. Novikov (1937) observed a marked decrease in both protein and soluble nitrogen in leaves of lucerne infected with the rust *Uromyces striatus*. He considered translocation to other parts of the plant unlikely and loss as gaseous nitrogen therefore probable, but gave no conclusive evidence for it.

Effect of type of nitrogen supply on non-nitrogenous substances in plants. Clark (1936) observed in tomatoes supplied with nitrate much higher contents of oxalic, malic and citric acids than in those supplied with ammonium salts. Similar data are reported for corn (*Zea mays*) (Wadleigh & Shive, 1939) and *Nicotiana rustica* (Vladimirov, 1939, 1945). Finch, Jones & van Horn (1945) found an inverse relation between nitrogen supply (as ammonium sulphate) and ascorbic acid content of fruits of cantaloupe and pepper

(*Capsicum* sp.), leaves and tubers of potato and leaves of *Tribulus terrestris*. Jones *et al.* (1944) got similar results for grapefruit and Wittwer, Schroeder & Albrecht (1945) for spinach. Acids other than ascorbic are not considered in these studies, but Hamner & Nightingale (1946) found a high correlation between ascorbic acid and total acidity in pineapple. Wittwer, Albrecht & Goff (1946) found with increased supply of nitrogen (as ammonium nitrate) an increase in the typically high oxalate content of Swiss chard. In mixed plantings of grasses and clover increased supplies of nitrogen, especially as nitrate, lead to an increase, significantly correlated with nitrate accumulation, in organic acid (Blackman & Templeman, 1940). Vickery, Pucher, Wakeman & Leavenworth (1940) report striking results with tobacco grown with ammonium as 0, 20, 40, 60, 80 and 90% of its nitrogen supply, the rest being nitrate. The total nitrogen supply was adequate for good growth but provided no excess. With 60% of the total nitrogen supply as ammonium, the content of malate was 2%, of citrate 4% and of oxalate 67% of those with nitrate alone. There was little change in unknown acids. Total soluble nitrogen, asparagine and glutamine were little affected by increasing ammonium supply, so acid production had not been inhibited by diversion of carbon to amides. Water content per plant and per unit fresh weight increased markedly with the proportion of ammonium. High-ammonium plants had less soluble carbohydrate, as in pineapple (Sideris, Krauss & Young, 1938*a*) (Arnon (1939) found the reverse in barley-leaf sap), and slightly higher pH. Organic acid therefore does not here determine sap pH. In pineapple, nitrate gives much more titratable acidity than ammonium but the pH is unaltered (Sideris, Krauss & Young, 1939).

In tobacco (Vickery *et al.* 1940) and pineapple (Sideris & Krauss, 1937) marked variations in plant composition can be induced without affecting growth or general health. Field plants fertilized with ammonium sulphate have organic acid contents corresponding to nitrogen supply mainly as nitrate, and store much nitrate; ammonium added to the soil is thus largely converted to nitrate before entering the plant. Sideris *et al.* (1939) got similar results with pineapple. In *Bryophyllum calycinum* increase in the proportion of nitrogen supplied as ammonium decreases malic, citric and isocitric acids and total acid. Glutamine increases up to a certain level of ammonium, then decreases; asparagine reverses this behaviour (Pucher *et al.* 1947*b*). Bulbs of *Narcissus poeticus* grown in culture solution form more of both amides with nitrate than with ammonium (Vickery *et al.* 1946).

The fungus *Armillaria mellea* forms crystals of calcium oxalate in culture when supplied with glucose and peptone in the ratio 4 : 1, but not at any of three lower ratios tested (Hamada, 1940). This might be due to the smaller supply of a reduced form of nitrogen, but more probably to the increased carbohydrate. Carotenoid production by *Mucor hiemalis* is sensitive to the carbon-nitrogen ratio of the medium (Schopfer, 1943). *Aspergillus niger* supplied with glucose but no nitrogen produces much pyruvic acid, which in cultures supplied with ammonium chloride is partially replaced by its dimethyl derivative (Hida, 1941).

The possible importance of nitrate as an oxidizing agent in plants has been stressed (Warburg & Negelein, 1920; Meyerhof, 1924; Arnon, 1937; Vladimirov, 1945). Burström (1945) criticizes the detailed interpretation of Warburg & Negelein's data but retains the conclusion that in comparing nitrate and ammonium as nitrogen sources their oxidation-reduction properties should not be forgotten. We have seen that nitrogen supply as nitrate favours production of organic acids; Vladimirov (1945) states that ammonium favours production of such reduced products as rubber (in *Taraxacum kok-saghyz*), pyrethrins and essential oils.

Cotton seedlings grown at low (0–15 %) oxygen tensions take up nitrate more readily than ammonium, but their organic nitrogen content and rate of root elongation are unaffected by the form of nitrogen supply (Leonard & Pinckard, 1946). Barley plants grow better with nitrate than with ammonium, but the difference can be eliminated by a high rate of aeration (Arnon, 1939). Decreased anthocyan formation with increased nitrate supply (Griffith, 1943; Woodman, 1939) is an exception to this relation, anthocyans being oxidized compounds. Wynd (1942) reports increased ascorbic acid production by Sudan grass with rising ammonium supply. There are errors of tabulation in this paper, but, allowing for these, ascorbic acid content per unit dry weight increases in some cases. Conversion of ammonium to nitrate before being taken up by the plants is not, however, excluded. Sideris, Krauss & Young (1938*b*) found that nitrate-grown pineapple plants had, in mature leaves, much more hexosamine than ammonium-grown; young leaves showed no differential effect.

Barley roots from nitrate plants have much more manganese, more calcium, magnesium and potassium and less phosphorus than those from ammonium plants (Arnon, 1939). In pineapple total ash per plant increases with nitrogen supply in the nitrate series and decreases in the ammonium series. Potassium and magnesium are independent of nitrate level but decrease with increasing ammonium. Calcium is related directly to nitrate and inversely to ammonium level. Phosphorus increases with nitrogen level in both series, but more is taken up with ammonium (Sideris & Young, 1945, 1946). Species differences or conversion of ammonium to nitrate in the soil may account for positive correlations (Boynton & Compton, 1944) between nitrogen, calcium and magnesium in leaves of apple trees fertilized with ammonium sulphate. Bean plants supplied with nitrogen as nitrate are much more severely affected by calcium deficiency than those with urea, though with adequate calcium nitrate gives better growth (Skok, 1941).

MISCELLANEOUS EFFECTS OF NITROGEN SUPPLY ON PLANTS

Many authors have used criteria other than changes in composition to assess the effects on plants of varied nitrogen supplies. Peach (Addoms, Nightingale & Blake, 1930; Nightingale, Addoms & Blake, 1930; Blake, Davidson, Addoms & Nightingale, 1931) and grapefruit (Martin, 1939) trees with high nitrogen supplies give large crops of fruit of poor eating quality. In peaches this is associated with lowered sugar, increased soluble organic nitrogen, later fruiting and almost complete suppression of the red skin pigment. The last effect is also reported in apples (Magness, Batjer & Regeimbal, 1939). Chapman, Brown & Liebig (1943) state that the coarse thick-skinned grapefruit considered typical of high nitrogen supply is associated rather with high nitrogen/phosphorus ratios; commercially acceptable fruit can be produced with high nitrogen supplies if the phosphorus supply is high also.

High nitrogen supply has generally been considered to prolong the vegetative period in plants. Garner, Bacon, Bowling & Brown (1934) noted, however, earlier flowering with rising nitrogen supply in tobacco, and more recent work (Chailakhyan & Lukovnikov, 1941; Chailakhyan, 1944; Vsevolozhskaya, 1946) has shown that flowering in different species may be retarded, accelerated or unaffected by rising nitrogen supply.

High nitrogen supply usually increases susceptibility of plants to pathogens (Wingard, 1941; Shear & Wingard, 1944; Walker & Foster, 1946), but some exceptions are known. McClure & Robbins (1942) found nitrogen-deficient cucumber seedlings more susceptible to the damping-off fungus, possibly through defective lignification of cell walls. Susceptibility of sugar beet to *Sclerotium rolfsii* is progressively reduced by increased applications of several nitrogenous fertilizers (Leach & Davey, 1942). Changing environmental factors may complicate the effects of nitrogen supply on disease development (Schroeder & Walker, 1942; Walker & Hooker, 1945*a*). Susceptibility of cabbage to *Plasmodiophora brassicae* is increased by both excess and deficiency of nitrogen compared with a balanced nutrient supply (Walker & Hooker, 1945*b*). McNew & Spencer (1939) showed that *Phytomonas stewartii*, the wilt bacterium of sweet corn (*Zea mays*), depends on inorganic nitrogen in the tracheal sap, where it lives during the early stages of its invasion. Intensity of infection is directly correlated with nitrogen content of the sap.

Rishkov & Smirnova (1939) report that virus formation in tomato plants is unaffected by nitrogen starvation. This is contradicted by Spencer (1941), whose nitrogen-deficient tomato seedlings had constant contents of virus and total protein over a period when both increased fivefold in controls with adequate nitrogen. He found that virus protein was not utilized in severe nitrogen deficiency. Takahashi (1941) similarly reports virus multiplication in detached darkened tobacco leaves during active hydrolysis of host proteins. Virus protein is thus not a respiratory substrate; work on detached leaves has not made clear whether nucleoprotein in general is so used. Bald (1942) suggests that virus *X* reduces yield in potatoes through diversion to virus protein of reserves for tuber production. Woods & Du Buy (1941) state that virus multiplication requires a cyanide-sensitive respiratory enzyme in the leaf. Prolonged nitrogen starvation partially inactivates this, so reducing virus infection. Lugg & Best (1945) found a progressive increase in precipitability in proteins of virus-infected tobacco leaves, though their amino-acid composition was unchanged.

Some insects attack preferentially nitrogen-low and others nitrogen-high plants (Haseman, 1946).

METABOLIC SIGNIFICANCE OF ORGANIC ACIDS

My earlier review (McKee, 1937) treated nitrogen metabolism as a set of reactions which, though clearly linked to carbohydrate metabolism by coupled reactions and some common intermediates, was essentially independent. All the metabolic activities of an organism are obviously connected and integrated; isolation of a particular field can thus be justified only by convenience of description of a complex set of reactions. Such widely used phrases as 'nitrogen metabolism' and 'carbohydrate metabolism' have served a useful purpose, and still may, if their arbitrary nature is clearly realized. Some phases of the transformations of nitrogenous substances in the plant can be adequately described without reference to other types of metabolism, but there is a large group of reactions where this is no longer possible. The point has been well expressed by Chibnall (1939): 'Proteins, carbohydrates and organic acids are all three intimately connected with the living processes of plants, and the metabolism of one of them cannot be satisfactorily investigated if concomitant changes in either or both of the other two are ignored', and 'My reading of a very extensive, if scattered, literature has impressed me with a firm belief that it is the group of substances we refer to as "organic acids"—which have in the past been neglected in studies of plant metabolism because satisfactory methods of characterisation were not available—that occupy the central, and therefore the key position, in the carbohydrate, protein and fat metabolism of plant cells.'

The tricarboxylic acid cycle. It is therefore necessary to review briefly present knowledge of organic acid metabolism. Excellent extensive accounts of the subject in all its complexity are given by Chibnall (1939), H. G. Wood (1946) and Braunstein (1947), the first and last laying particular emphasis on the connexions between organic acids and nitrogenous compounds. Much of the evidence now available refers to animal tissues, which have, in this field as in so many others, been studied more than those of plants, but Vickery *et al.* (1939, 1940, 1946) of New Haven, U.S.A., and J. G. Wood (1941; see also J. G. Wood, Cruickshank & Kuchel, 1943; and J. G. Wood, Mercer & Pedlow, 1944) of Adelaide, Australia, give extensive data for several plant species and discuss the theoretical implications.

Connexion with carbohydrate metabolism. Krebs & Johnson (1937) suggested that a citric acid cycle plays a major part in carbohydrate oxidation in the animal. In a later modification Krebs (1943) considers citric acid a side product and speaks therefore of a tricarboxylic acid cycle. A more recent formulation (H. G. Wood, 1946) (Fig. 1) substitutes for pyruvate in the condensation of oxalacetate to aconitate a C_2 compound whose exact nature is still unknown. It is usually represented as 'active acetate' or $CH_3.COX$. Condensation of acetate and oxalacetate to form citrate was suggested much earlier by Raistrick & Clark (1919).

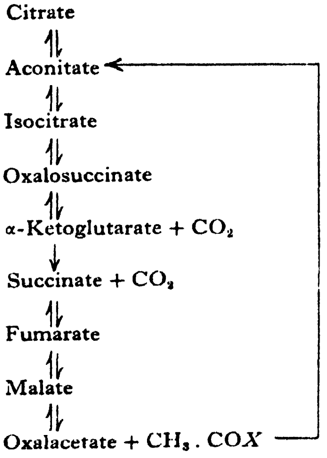


Fig. 1

The original evidence for the cycle was based on observations that succinate, fumarate, malate, oxalacetate (Szent-Györgyi, 1936; Stare & Baumann,. 1936) and citrate, isocitrate and *cis*-aconitate (Krebs & Johnson, 1937) all catalyse carbohydrate oxidation in animal tissues, and citrate is synthesized from oxalacetate. Martius (1937, 1938), using bacteria, animal tissues and cucumber seed extracts, showed that citric, isocitric and *cis*-aconitic acids are biologically equivalent through reversible reactions.

Connexion with fat metabolism. Breusch (1943) reports, without experimental data, isolation from animal tissues of an enzyme citrogenase which condenses β-keto-acids, derived from fatty acids, with oxalacetate to form citrate, as below:

$$R.CO.CH_2.COOH + H_2O + COOH.CO.CH_2.COOH$$
$$= R.COOH + COOH.CH_2.C(OH).COOH.CH_2.COOH.$$

The fatty acid $R.COOH$ can undergo further β-oxidation and condensation until completely oxidized. Breusch maintains the apparently illogical position that fat but not carbohydrate is oxidized by the tricarboxylic acid cycle. Formation of citrate by kidney from acetoacetate and oxalacetate is reported by Wieland & Rosenthal (1943), and by yeast from acetate and oxalacetate (Lynen, 1943). Krebs & Eggleston (1945) deny, however, that formation of citrate from acetoacetate is an important metabolic path in animal tissues and show that acetoacetate metabolized anaerobically in sheep heart can nearly all be accounted for as β-hydroxybutyrate. They suggest the primary reaction

$$\text{malate} + \text{acetoacetate} = \text{oxalacetate} + \beta\text{-hydroxybutyrate},$$

citrate being formed indirectly from oxalacetate. In aerobic conditions, however, labelled carbon from acetoacetate is transferred in significant amounts to fumarate, succinate and α-ketoglutarate (Buchanan, Sakami, Gurin & Wilson, 1945). In rat heart muscle, too, succinate production from caprylate and acetoacetate agrees quantitatively with the hypothesis that C_2 fragments of the fatty acid condense with oxalacetate and are further metabolized via the tricarboxylic acid cycle (Lehninger, 1946).

The most obvious gap in current knowledge of organic acid metabolism concerns the nature of the C_2 compound condensing with oxalacetate and especially its connexion with acetate. The metabolic importance of acetate is stressed by the extreme toxicity to plant and animal tissues of fluoracetate, which competitively inhibits acetate oxidation (Bartlett & Barron, 1947). Fluoracetate, though without effect on formation of alanine or lactate from pyruvate, impedes utilization of the latter, whose metabolism must therefore involve acetate. Inhibition by fluoracetate of fat oxidation implies that it also is closely connected with acetate metabolism. Further study of the metabolism of C_2 compounds should clarify both the relation of the tricarboxylic acid cycle to fat metabolism and the least known step of the cycle itself. Current knowledge of acetate metabolism in animal tissues is reviewed by Bloch (1947).

Fixation of carbon dioxide in organic acids. The organic acids are associated also with the fixation, first demonstrated by H. G. Wood & Werkman (1936, 1938) with propionic acid bacteria, of carbon dioxide by heterotrophic organisms. Fixation of labelled carbon dioxide by enzyme preparations from *Micrococcus lysodeikticus* (Krampitz, Wood & Werkman, 1943) follows the reaction

$$\mathrm{C^{13}O_2} + \underset{\text{Pyruvic acid}}{\mathrm{CH_3.CO.COOH}} \rightleftharpoons \underset{\text{Oxalacetic acid}}{\mathrm{C^{13}OOH.CH_2.CO.COOH}},$$

later demonstrated in animal tissues also (Evans, Vennesland & Slotin, 1943; Utter & H. G. Wood, 1945). H. G. Wood quotes unpublished work by Wilson, Krampitz & Werkman showing that a cell-free extract of *Clostridium butylicum* fixes carbon dioxide as pyruvate, acetyl phosphate being a probable intermediate. Fixation of carbon dioxide in α-ketoglutaric acid is coupled with a glucose-6-phosphate dehydrogenase system to give the reaction

$$\alpha\text{-ketoglutarate} + \mathrm{CO_2} + \text{glucose-6-phosphate} = \text{isocitrate} + \text{6-phosphogluconate}$$

(Ochoa, 1945; Ochoa & Weisz-Tabori, 1945). Vennesland, Ceithaml & Gollub (1947) report a similar fixation in plant material. An enzyme from pigeon liver catalyses the reaction

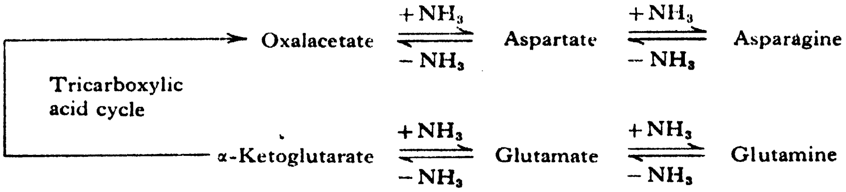
$$\mathit{l}\text{-malic acid} + \mathrm{TPN_{ox.}} \rightleftharpoons \text{pyruvic acid} + \mathrm{CO_2} + \mathrm{TPN_{red}}$$

(TPN = triphosphopyridine nucleotide) (Ochoa, Mehler & Kornberg, 1947).

These fixations of carbon dioxide suggest a comparison with photosynthesis. Ruben, Hassid & Kamen (1940*b*), using C^{11} with the green alga *Chlorella pyrenoidosa*, showed that carbon dioxide is taken up both in the light and the dark, the reduction products having many common properties. Much of the radioactivity was in carboxyl groups, but no definite acid could be identified as carrying them. Ascorbic, citric, malic, fumaric, maleic, succinic, oxalic and tartaric acids were excluded.

A better knowledge of the enzyme systems catalysing each step in the cycle would do much to clarify details of its operation and modification in different tissues. Such knowledge is far from complete for plants, but Vennesland & Felsher (1946) found oxalacetate carboxylase activity in crystalline globulins from various cucurbit seeds and independent oxalacetate and pyruvate carboxylases in parsley roots. Gollub & Vennesland (1947), using C^{14}, demonstrated catalysis of carbon dioxide fixation by the parsley enzyme, and suggested that dicarboxy-acids may arise in plants, as in bacteria and animal tissues, via an initial formation of oxalacetate from carbon dioxide and pyruvate. Thiamin, from which cocarboxylase is formed by phosphorylation, is probably essential to plant cells; biotin, equally widely distributed, seems directly involved in oxalacetate synthesis (Stokes, Larsen & Gunness, 1946; Lardy, Potter & Elvehjem, 1947; Shive & Rogers, 1947).

Connexions with nitrogen metabolism. The tricarboxylic acid cycle thus draws together the catabolism of carbohydrate and of fat. Certain of its members are substrates for carbon dioxide fixation; some are definitely, others probably connected with the phosphorylations known (Lipman, 1946) to play a major part in metabolic energy transfer. Oxalacetic and α-ketoglutaric acids are readily aminated to aspartic and glutamic acids, whose central position in nitrogen metabolism is clear from their rapid formation (see p. 25) when nitrogen in any form enters the plant cell. Their importance is further suggested by their occurrence in large amounts in seed reserve proteins and by their close relations with the amides asparagine and glutamine. The main links between the cycle and nitrogen metabolism are shown diagrammatically below:



Braunstein (1947) emphasizes the metabolic versatility conferred upon the cell by the varied reactions involving the interconvertible acids. He writes: 'Each conversion of a dicarboxylic acid into another link of the cycle automatically blocks certain acceptor or carrier functions and opens other metabolic circuits. Thus, the reaction oxalacetate → malate brings into action catalytic hydrogen transfer and blocks reversible decarboxylation and amino-nitrogen transfer. Formation of glutamate from α-ketoglutarate opens the way for amidation, while it renders the dicarboxylate molecule inactive in CO_2 fixation or oxidative phosphorylation and interferes with the Krebs cycle. The conversion of glutamate to glutamine or of aspartate to asparagine stops transamination, and so on, all these interrelations being reciprocal. The implications with respect to metabolic co-ordination are self-evident. The shunting effects of shifts in the dicarboxylic acid system afford exceedingly manifold possibilities for the mutual automatic control of various phases of nitrogen metabolism and cellular respiration.'

Evidence that the tricarboxylic acid cycle operates in plant tissues is still scanty, but its use in discussion of their results by three leading workers on intermediate plant metabolism (Chibnall, Vickery, J. G. Wood) shows its pragmatic value. Boswell (1945) has demonstrated in potato-tuber slices an oxidation system involving malic, succinic, fumaric, oxalacetic and pyruvic acids.

Survey of plant acids. Oxalosuccinic is the only acid involved in the tricarboxylic acid cycle which has not been reported in plants. Citric and malic are widespread, aconitic is reported in wheat (Nelson & Hasselbring, 1931) and in considerable quantity in sugar cane (Balch, 1945), succinic in several species (Franzen & Stern, 1922; Franzen &

Helwert, 1922; Pucher & Vickery, 1941), α-ketoglutaric and oxalacetic in legumes (Virtanen *et al.* 1943), fumaric in *Fumaria officinalis* (Karrer, 1938) and isocitric as the main acid, formerly known as 'Crassulacean malic acid', of the leaves of *Bryophyllum calycinum* (Pucher & Vickery, 1942).

Other acids, of unknown relationship to the tricarboxylic acid cycle, occur in plants. Oxalic acid is widespread, and in many plants a major component. It might arise by oxidation of acrylic acid (see p. 36) or of glyoxylic acid (see p. 58). Saturated unsubstituted dicarboxylic acids known from plants include malonic (C_3), a specific inhibitor of succinic dehydrogenase, in wheat (Nelson & Hasselbring, 1931) and sugar beet, glutaric (C_5) and adipic (C_6) in sugar beet and pimelic (C_7) in castor oil from *Ricinus communis* (Karrer, 1938). Lactic acid occurs in raspberry leaves and cherry fruits (Franzen & Stern, 1922; Franzen & Helwert, 1922). It has recently been demonstrated in many species and shown to be an active metabolite during germination (Schneider, 1942). Tartaric is the main acid of grapes (*Vitis vinifera*); Franzen & Helwert (1923), reviewing its reported occurrence, reached the somewhat salutary conclusion that in five of the eighty-two species in which it was reported its presence was established and in one more it was probable. Ascorbic acid has been found in all plant tissues examined for it except dry seeds, some mosses (Tuba, Hunter & Osborne, 1946) and the total parasites *Lathraea* and *Orobanche* (Moldtmann, 1940), but is structurally more related to the sugars than to the other organic acids. It is stated (Rubin *et al.* 1946) to be synthesized directly from glucose in the pericarp of fruits of *Rosa spinosissima* and *R. cinnamomea.*

Even within a single species organic acid contents can vary widely. Kostina (1936) records total acid contents ranging from 1·2 to 12·8% of the dry weight in the fruit of thirty-seven apricot varieties grown in Central Asia. In *Nicotiana tabacum* and *N. rustica* tetraploid plants have 1·3 to 2·2 times the organic acid content of diploids (Noguti, Oka & Ôtuka, 1940).

Formation of amino-acids from keto-acids. Knoop & Oesterlin (1925) showed that *in vitro* oxalacetic and α-ketoglutaric acids form, with ammonia, imino-acids readily reduced to aspartic and glutamic acids. Their suggestion that this occurs *in vivo* also was confirmed by Adler, Das, von Euler & Heyman (1938), who showed that dihydrocodehydrase reduces iminoglutaric acid to glutamic acid. Adler, von Euler, Günther & Plass (1939) demonstrated in animal tissues the sequence

isocitric acid → α-ketoglutaric acid → α-iminoglutaric acid → glutamic acid.

TRANSAMINATION

There is much evidence for a key role of aspartic and glutamic acids in amino-acid metabolism. Work with N^{15} has shown that *Azotobacter* supplied with free nitrogen or with ammonia (Burris, 1942; Burris & Wilson, 1946*a*), rats fed with ammonium citrate or amino-acids (Foster, Schoenheimer & Rittenberg, 1939; Rittenberg *et al.* 1939) and tobacco plants given ammonium chloride (Vickery *et al.* 1940) accumulate the heavy isotope first in glutamic and aspartic acids and later in other nitrogen fractions. Liver slices incubated for ½–3 hr. in bicarbonate labelled with C^{11} incorporated radioactive carbon in their protein, nearly all in glutamic and aspartic acids (Anfinsen, Beloff, Hastings & Solomon, 1947). C^{13} fed to intact animals as labelled acetate is deposited in aspartic and glutamic acids (Rittenberg & Bloch, 1945).

Glutamic and aspartic acids owe much of their metabolic versatility to the transamination reaction, first discovered by Braunstein & Kritzmann (1937*a*, *b*), whereby amino-groups are transferred from one molecule to another. The most studied transamination reactions are

$$\text{glutamic acid} + \text{pyruvic acid} \rightleftharpoons \text{alanine} + \alpha\text{-ketoglutaric acid}$$

(Braunstein & Kritzmann, 1937*a*, *b*);

$$\text{glutamic acid} + \text{oxalacetic acid} \rightleftharpoons \text{aspartic acid} + \alpha\text{-ketoglutaric acid}$$

(Banga & Szent-Györgyi, 1937; Cohen, 1939*b*);

$$\text{aspartic acid} + \text{pyruvic acid} \rightleftharpoons \text{alanine} + \text{oxalacetic acid}$$

(Karyagina, 1939; Cohen & Hekhuis, 1941). O'Kane & Gunsalus (1947) state that the third of these reactions arises by combined action of the enzymes catalysing the other two.

Most work on transamination has been done with animal tissues, but Virtanen & Laine (1938) demonstrated transfer of an amino-group from aspartic acid to pyruvic acid in pea tissue. Kritzmann (1939) prepared from plant tissues an aminopherase specific for aspartic acid and without action on glutamic acid. Rautanen (1946), with plant material of unstated species, found transamination quicker and more complete with glutamic acid or α-ketoglutaric acid as the dicarboxylic component than with aspartic acid or oxalacetic acid. Albaum & Cohen (1943) demonstrated transamination between glutamic and oxalacetic acids in developing oat embryos, and Virtanen & Laine (1941), with pea shoots, found transamination most effective with glutamic acid. They found a slight effect with glutamine but concluded, in agreement with Braunstein (1939), that amides probably take part in transamination via the corresponding dicarboxy-acids. Leonard & Burris (1947) found aminopherases in all of twenty-two species examined. Damodaran & Nair (1938) found in seedlings of pea, *Phaseolus mungo* and *P. radiatus*, an aerobic dehydrogenase forming α-ketoglutaric acid and ammonia from glutamic acid. It was apparently absent from seedlings of nine other species, mostly legumes.

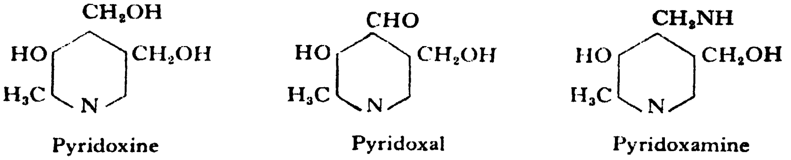
Ochoa (1944) showed that α-ketoglutarate is formed in animal tissues from carbohydrate via pyruvate and isocitrate, from glutamate by oxidative deamination or by transamination, and from fatty acids via enzymatic condensation of oxalacetate with β-keto-acids to citrate. It is readily oxidized by most animal cells to carbon dioxide and water, but succinate and carbon dioxide are formed when succinic dehydrogenase is blocked by malonate. Krebs & Cohen (1939) demonstrated in rat and pigeon tissues the reaction

$$2\alpha\text{-ketoglutaric acid} + \text{ammonia} = \text{glutamic acid} + \text{succinic acid} + CO_2,$$

and considered it a probable step in the normal oxidative breakdown of α-ketoglutarate. Felix & Naka (1940) found rapid breakdown of glutamic and aspartic acids by kidney slices, only one-third of the ammonia expected on complete oxidative deamination being formed. Glutamic acid took up $2O_2$ per molecule and produced $2CO_2$, and aspartic one of each. The authors suggest both are broken down to the same C_3 amino-compound. *Bacterium coli* (Adler, Hellström, Günther & von Euler, 1938) and yeast (Adler, Günther & Everett, 1938) fix ammonia by amination of α-ketoglutaric acid and transamination of the glutamic acid so formed with other α-keto-acids.

Braunstein (1947) gives a very clear account of the detailed mechanism of transamination and discusses similarities and differences between the enzyme-catalysed reactions and the non-biological reaction studied by Herbst & Rittenberg (1943). Enzymatic, but not non-biological transamination involves dissociation of the α-hydrogen of the original amino-acid and its replacement, in the amino-acid formed, by hydrogen from the aqueous reaction medium (Konikova, Dobbert & Braunstein, 1947). Bychkov (1939) showed that the acid-substituted amino-acids, cysteic acid ($SO_3H.CH_2.CHNH_2.COOH$) (confirmed by Cohen, 1940) and phosphoserine ($H_2PO_3.O.CH_3.CHNH_2.COOH$) act in transamination like the dicarboxy-acids. Knoop & Martius (1938), in their synthesis of octopine, found in many marine animals, demonstrated a condensation in aqueous medium of arginine, which does not take part in transamination, and a keto-acid. The enzymes involved in transamination have been variously termed aminopherases and transaminases; the former name has priority, and Braunstein (1947) gives adequate reasons for its retention.

Vitamin B_6 occurs naturally in three forms, pyridoxine, pyridoxal and pyridoxamine (Snell, 1944, 1945*a*) (see Fig. 2); there is much evidence that, as suggested by Schlenk & Snell (1945), its derivative pyridoxal phosphate (synthesized by Gunsalus, Umbreit, Bellamy & Foust (1945) is associated with aminopherase. Lichstein, Gunsalus & Umbreit (1945) and Green, Leloir & Nocito (1945) consider that pyridoxal phosphate is its coenzyme; Schlenk & Fisher (1945) showed that vitamin B_6 and enzyme activity are correlated. Lyman, Moseley, Wood, Butler & Hale (1947) found that lactic acid bacteria require vitamin B_6 for synthesis of aspartic acid. Ames, Sarma & Elvehjem (1947) showed a marked decrease in aminopherase activity in B_6-deficient rats, and Bellamy, Umbreit & Gunsalus (1945) a direct relation between the coaminopherase content of rat tissue and the pyridoxine level of the diet. Snell (1945*b*) demonstrated *in vitro* the reaction



Pyridoxine Pyridoxal Pyridoxamine

Fig. 2

$$\text{pyridoxal} + \text{glutamic acid} \rightleftharpoons \text{pyridoxamine} + \alpha\text{-ketoglutaric acid.}$$

Schlenk & Fisher (1947) cite evidence of a similar change in the prosthetic group during enzymatic transamination.

In spite of all this evidence it is still not certain (Braunstein & Kritzmann, 1946; Gale & Tomlinson, 1946) that pyridoxal phosphate alone is sufficient to activate aspartic apo-aminopherase. Kritzmann & Samarina (1946) found that pyridoxal phosphate activated glutamic aminopherase at low and inhibited it at higher concentrations.

Pyridoxal phosphate is also a coenzyme for some, but not all, of a group of amino-acid decarboxylases (Umbreit & Gunsalus, 1945; Gale & Epps, 1944*a*, *b*; Baddiley & Gale, 1945; Taylor & Gale, 1945; Epps, 1945; Schales, Mims & Schales, 1946; Schales & Schales, 1946) and for enzymes, isolated from *Neurospora sitophila* and *Escherichia coli* respectively, catalysing the reactions

$$\text{indole} + \text{serine} \rightarrow \text{tryptophane} \quad \text{and} \quad \text{tryptophane} \rightarrow \text{indole} + \text{pyruvic acid} + \text{ammonia}$$

(Umbreit, Wood & Gunsalus, 1946; W. A. Wood, Gunsalus & Umbreit, 1947).

Livers of rats deficient in thiamin, riboflavine or pyridoxine all show a marked increase in histidase and arginase activity (Edlbacher & Viollier, 1943). The authors suggest that the vitamin deficiencies disturb carbohydrate metabolism and so reduce production of oxalacetate, which in compensation is formed via histidine → glutamic acid → α-ketoglutaric acid → oxalacetic acid. There appear to be two pathways of histidine breakdown in the vertebrate liver, by histidase with opening of the imidazole ring to form formylglutamine, and by α-deamination giving urocaninic acid (imidazoleacrylic acid); both lead eventually to glutamic acid (Edlbacher & von Bidder, 1942; Edlbacher & Heitz, 1942). An increased synthesis of urea was also noted; it may be connected with the necessity (Cohen & Hayano, 1946*a*, *b*) for glutamic acid in the conversion of citrulline to arginine in liver and kidney, which also requires adenosine triphosphate, thus coupling the endergonic urea cycle with exergonic oxidation-reduction systems. A connexion of glutamic acid with urea metabolism is also indicated by its continuous production in normal mice from ornithine via arginine and proline (Roloff, Ratner & Schoenheimer, 1940). Dietary deficiencies not affecting carbohydrate metabolism do not increase histidase activity. The effects of pyridoxine deficiency on other enzymes may be complicated by its connexion, mentioned above, with aminopherase activity.

SYNTHESIS OF AMINO-ACIDS

It is generally accepted that reductive amination of α-keto-acids followed by transamination is a major pathway of amino-acid formation. These processes account for formation of the dicarboxy-amino-acids, whose primary role has already been stressed, and of alanine, from which all other naturally occurring amino-acids except proline and hydroxyproline may, formally at least, be derived by substitution. Recent work shows that details of the synthesis may be more complex than was at first supposed. Kritzmann & Melik-Sarkisyan (1945*a*, *b*) found that rat liver produces aspartic acid from pyruvic acid and ammonia after a short incubation, and alanine later. The synthesis requires carbon dioxide but can be induced in its absence by small amounts of oxalacetate, α-ketoglutarate, their metabolic precursors (malate, fumarate, succinate, citrate) or, less effectively, by their lower (mesoxalate) or higher (α-ketoadipate) homologues, which are also transamination substrates. Phosphate is also required, probably in the synthesis of oxalacetate. Lyman *et al.* (1947) report that *Lactobacillus arabinosus* requires carbon dioxide and pyridoxine for synthesis of aspartic acid, arginine, phenylalanine and tyrosine, suggesting oxalacetic acid as their precursor. They also got evidence that reversal of the amino-acid decarboxylase reaction may be one pathway of amino-acid synthesis. Kritzmann (1947) suggests the following scheme for amino-acid synthesis:

(1) $CH_3.CO.COOH + CO_2 \rightarrow HOOC.CH_2.CO.COOH$ (phosphate required);

(2) $HOOC.CH_2.CO.COOH + NH_3 + H_2X \rightarrow HOOC.CH_2.CHNH_2.COOH + H_2O + X$;

(3) $HOOC.CH_2.CHNH_2.COOH + CH_3.CO.COOH \rightarrow CH_3.CHNH_2.COOH + HOOC.CH_2.CO.COOH$;

(2)+(3) $CH_3.CO.COOH + NH_3 + H_2X \rightarrow CH_3.CHNH_2.COOH + H_2O + X$.

(X = unknown hydrogen donator.)

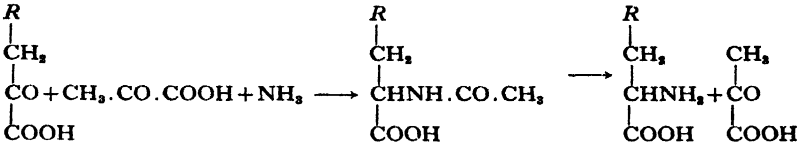
α-Ketoglutaric acid and glutamic acid can replace oxalacetic acid and aspartic acid in this scheme. The reverse process, oxidative indirect deamination of amino-acids by transfer of amino-groups to α-ketoglutaric acid to form glutamic acid, later deaminated by glutamic dehydrogenase, is also known (Braunstein & Asarkh, 1945). Kaplanski & Shmerling

(1945) claim, however, that the rate of formation of amino-acids by liver and kidney slices is independent of bicarbonate supply; the reason for this disagreement with Kritzmann's data is not clear.

Burström (1943*a*, *b*) showed that green leaves require light and carbon dioxide for assimilation of nitrate; at high light intensities, with carbon dioxide presumably the limiting factor, protein synthesis reduces that of sugar. These data suggest initial formation, in photosynthetic as well as heterotrophic fixation of carbon dioxide, of keto-acids leading either to carbohydrate or to amino-acids.

Nitrogen-starved *Torula utilis* when amply supplied with ammonia accumulates first glutamic and aspartic acids, then alanine, suggesting active transamination (Roine, 1946*a*, *b*).

The early theory that acetyl derivatives are intermediates between keto- and amino-acids, as below, has been revived by du Vigneaud & Irish (1938):



$$\begin{array}{l} R \\ | \\ CH_2 \\ | \\ CO \\ | \\ COOH \end{array} + CH_3.CO.COOH + NH_3 \longrightarrow \begin{array}{l} R \\ | \\ CH_2 \\ | \\ CHNH.CO.CH_3 \\ | \\ COOH \end{array} \longrightarrow \begin{array}{l} R \\ | \\ CH_2 \\ | \\ CHNH_2 \\ | \\ COOH \end{array} + \begin{array}{l} CH_3 \\ | \\ CO \\ | \\ COOH \end{array}$$

This suggestion is supported by the finding (Bloch & Borek, 1946) that rat-liver slices incubated with deuterio-acetic acid and leucine, phenylalanine or phenylaminobutyric acid form the corresponding deuterio-acetylamino-acids. Acetylphenylaminobutyric acid, not further metabolized, accumulates much more than the other compounds. This mechanism appears not to operate in *Escherichia coli*, where, for mutant strains unable to synthesize them, tyrosine and phenylalanine are replaceable by their simple peptides and by the corresponding keto-acids (phenylpyruvic and *p*-hydroxyphenylpyruvic), but not by acetyltyrosine and acetylphenylalanine (Simmonds, Tatum & Fruton, 1947).

Studying the inhibition of growth of *Escherichia coli* by hydroxyaspartic acid and its reversal by pantothenic acid and β-alanine, Shive & Macow (1946) concluded that this organism carries out the synthetic sequence: aspartic acid → β-alanine → pantothenic acid. Cysteic acid also prevents the synthesis of pantothenic acid by blocking some enzyme decarboxylating aspartic acid to β-alanine (Ravel & Shive, 1946). The malaria parasite, *Plasmodium gallinaceum*, incubated in chicken erythrocytes, produces much amino-nitrogen in presence of glucose, but in its absence accumulates ammonia (Moulder & Evans, 1946).

AMIDE METABOLISM

Animals. Recognition that glutamine is important in animals as well as plants dates probably from the observation (Thierfelder & Sherwin, 1914) that the human body uses it to conjugate ingested phenylacetic acid. Krebs (1935) showed that various mammalian tissues synthesize glutamine endothermically from ammonium glutamate by a specific glutaminase, and Örström, Örström, Krebs & Eggleston (1939) that avian livers form it from ammonium pyruvate. Ferdman, Frenkel & Silakova (1942) reported glutamine from several animal tissues, as was confirmed by Hamilton (1945). Van Slyke, Phillips, Hamilton, Archibald, Futcher & Hiller (1943) found glutamine a major source of urinary ammonia in the dog. Fertilized, in contrast to unfertilized, eggs of the sea urchin

Paracentrotus lividus (Örström, 1941) synthesize glutamine from ammonia and glutamic acid, and take it up about three times as rapidly from an external supply. The reaction

$$\text{glutamine} \rightleftharpoons \text{glutamic acid} + \text{ammonia}$$

is important in the active ammonia metabolism, linked apparently to glycolysis, of these eggs. Synthesis of glutamine from glutamic acid and ammonia in pigeon-liver extracts requires adenosine triphosphate and Mg^{++} ions and, for full activity, sulphydryl groups (Speck, 1947). Krebs (1942) confirms earlier observations that liver can synthesize urea rapidly from glutamine but considers the connexion indirect. Archibald (1945*a*) states that his earlier report of urea formation from glutamine was due to the presence of arginine, a frequent impurity difficult to detect, except by using a specific glutaminase. Örström, Örström & Krebs (1939) showed that glutamine and oxalacetate accelerate hypoxanthine synthesis in pigeon liver, suggesting that oxalacetate provides carbon for synthesis of the purine ring and that glutamine transfers ammonia.

Asparagine, unlike glutamine, has rarely been reported an animal metabolite. Ussing (1945) found appreciable amounts in the haemolymph of *Melolontha* larvae. Its presence in animal proteins is possible but not certain (Brand *et al.* 1945; Archibald, 1945*b*); the high asparaginase activity of some mammalian tissues (Krebs, 1935) suggests that they may metabolize it. Glutamine is important in storing ammonia and glutamic acid, whose central position in metabolism is clear, and the lability of its amide group may make it particularly effective in ammonia transfer.

Bacteria. Glutamine is an essential and very specific growth factor for haemolytic streptococci, which cannot form it from glutamic acid or hydrolyse peptide linkages between its carboxy group and the amino-groups of glycine, glutamic acid or cysteine. Production of ammonia from glutamine occurs only while glucose is being metabolized to lactic acid; glycolysis is stimulated by ammonia, but the effect of glutamine exceeds that of its ammonia equivalent (McIlwain, 1939, 1946*a*; McIlwain, Fildes, Gladstone & Knight, 1939). *Lactobacillus arabinosus* also requires glutamine (Hac, Snell & Williams, 1945) but can form it from glutamic acid; it uses asparagine only after conversion to aspartic acid (Hac & Snell, 1945).

Plants. Chibnall (1939) infiltrated *Lolium perenne* leaves with ammonium glutamate, glutarate, glutaconate and phosphate. All gave glutamine synthesis, so that use of the carbon of the organic acids in its synthesis could not be demonstrated. Rapid disappearance of infiltrated ammonium α-ketoglutarate and a careful balance sheet of changes in nitrogen fractions, ketonic and other organic acids, and carbohydrates made it reasonably certain as the true precursor.

J. G. Wood (1933) showed a marked decrease of protein, but no corresponding increase in amides, amino-acids or ammonia in darkened leaves of *Atriplex nummularium* attached to the shoot. Increase in amides in darkened leaves with their petioles in water was slight, possibly because of their characteristically low carbohydrate content. Leaves of *Phalaris tuberosa* supplied with sucrose externally showed increases in amino-acids, asparagine and glutamine with rising ammonia supply up to a point but not thereafter. External supply of asparagine led not to its accumulation but to formation of amino-acids and protein (J. G. Wood, 1942). In starving leaves of Sudan grass and kikuyu grass (*Pennisetum clandestinum*) amino-acids, glutamine, asparagine and ammonia reach maxima in that order (J. G. Wood *et al.* 1943) as in barley (Yemm, 1937; McKee, 1938) and several species examined by Mothes (1940). With kikuyu grass glutamine accumulation is small compared with that of asparagine. Leaves of Algerian oats (*Avena sterilis*) differ from those of kikuyu grass in more extensive protein breakdown, peptide accumulation and secondary glutamine formation (J. G..Wood & Cruickshank, 1944).

Rhubarb leaves held in the dark with their petioles in distilled water showed no protein loss in the blades for 48 hr., then rapid loss to 165 hr. and a steady protein content thereafter. The petioles maintained their protein content for 200 hr., loss of soluble carbohydrate plus malic acid accounting for their total loss in weight. The leaves when removed from the plant had up to 15% of their total nitrogen as ammonia, whose increase during the experiment was comparatively slight. In the petioles 50% to nearly 90% of the soluble nitrogen was ammonia. This high content of ammonium ion seemed neither to damage the leaf cells nor to stimulate glutamine synthesis (Vickery & Pucher, 1939; Vickery, Pucher, Leavenworth & Wakeman, 1938; Vickery *et al.* 1939).

Tobacco (*Nicotiana tabacum*) leaves cultured in water or nutrient solutions in the dark accumulated much asparagine and little glutamine. In light less amide was formed, but much of it was glutamine. Ammonia formed from nitrate and deamination of amino-acids formed in protein hydrolysis was equivalent to that used in amide synthesis plus free ammonia. Total organic acid showed little change during starvation. Malic acid in the dark, but not in light, was rapidly converted to citric acid (Vickery, Pucher, Wakeman & Leavenworth, 1937). This conversion is reported also in *N. rustica* and *N. glauca* by Piatnitski (1940*a*, *b*). Vickery *et al.* (1937) suggest that malic acid gives rise to oxalacetic acid which condenses with pyruvic acid to form citric acid. In kikuyu grass and Sudan grass (J. G. Wood *et al.* 1943, 1944) malic acid increases up to a late stage of starvation. Chibnall (1939) quotes unpublished data by Yemm & Somers showing in starving barley leaves a slight increase in citric acid and a roughly equivalent decrease in malic acid. The interrelationships of the organic acids as well as those of asparagine and glutamine thus vary in different species, as stressed by Pucher, Wakeman & Vickery (1938). Malic and citric acids, probably derived in part from amides, increase in starving oat leaves (Cruickshank & J. G. Wood, 1945).

Vickery & Pucher (1943) record that in etiolated seedlings of *Lupinus angustifolius* asparagine accumulated 12 days after germination to about 11% of the weight of the original seeds. It then broke down to ammonia and the plants collapsed. In *Vicia atropurpurea* seedlings the accumulation reached 5% of the original seed weight, and then remained steady till the end of the experiment at 26 days. *Cucurbita pepo* seedlings produced in 21 days glutamine to 3% of the original seed weight and about half as much asparagine. No nitrogen was supplied externally in these experiments. Burkhart (1938) records similar observations on etiolated seedlings of *Cucurbita pepo*, *Arachis hypogaea*, *Lupinus albus* and *L. luteus*; external ammonium supply increased carbohydrate consumption. Seedlings of *Zea mays* respond to a high ammonia supply by synthesis of glutamine, asparagine and amino-acids in the roots and their translocation to the tops (Viets, 1945; Viets, Moxon & Whitehead, 1946). McRary (1942) found that glutamic but not aspartic or oxalacetic acid increased asparagine production in embryos of *Lupinus hartwegii*. He suggests that energy supply limits synthesis and is provided by metabolism of glutamic acid. Ammonium supply had only a slight effect on amide synthesis. Asparagine accumulation in barley seedlings is increased by darkness compared with light and by ammonium compared with nitrate supply. Glutamine does not accumulate (McKee, 1938).

Exudation of glutamine and its deposition as a solid on the surface of leaves was observed by Curtis (1944) on *Rumex acetosella*, *Achillea millefolium*, *Trifolium repens*, *Hieracium pratense* and grass species. Raleigh (1946) induced exudation by heavy applications of ammonium chloride to nitrogen-low plants with high carbohydrate reserves.

Steward & Preston (1941*a*, *b*) and Steward & Street (1946) with potato tubers and Roine (1946*a*, *b*) with yeast obtained evidence that nitrogen for protein synthesis is canalized through glutamine. Said & el Shishiny (1944) found, however, a steady amide content during protein synthesis by radish-root disks. Stuart & Appleman (1935) state that amino-acids rather than amides provide nitrogen for protein formation in tissue regeneration by wounded potato tubers. Protein synthesis in stored apples, which lack glutamine, is at the expense of asparagine and amino-acids (Hulme, 1936).

In sugar-beet leaves exposed to 20 and 40% carbon dioxide ammonia increases and asparagine decreases; with 80 and 100% much ammonia is formed and asparagine increases. Some of the amide formed at high carbon dioxide concentrations seems to arise in peptide hydrolysis (Fife & Ferguson, 1941). In beet-tumour tissue glutamine replaces asparagine in anaerobiosis; it appears to be synthesized from malic acid (Neish & Hibbert, 1944). Neuberger & Sanger (1942) report that asparagine in potato tubers is located mostly in skin and cortex, and glutamine in the inner parts. Frond production by *Spirodela polyrhiza* (Lemnaceae) in controlled sterile conditions increases strikingly on addition of asparagine to the medium. Vitamins and sixteen amino-acids tested had no such effect (Gorham, 1945).

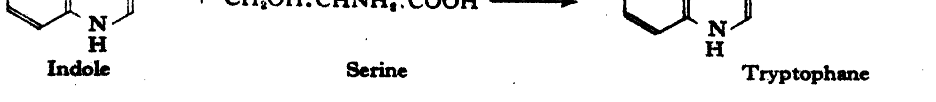
METABOLISM OF AMINO-ACIDS

Oxidative deamination of alanine (Edlbacher & Grauer, 1944*a*) and of several other amino-acids (Blanchard, Green, Nocito & Ratner, 1945) occurs in kidney. The enzyme responsible is a flavoprotein with riboflavin phosphate as its prosthetic group. Edlbacher & Grauer (1944*b*) challenge the common assumption that a single enzyme catalyses oxidative deamination of several amino-acids. They cite evidence from differential inhibition effects that in kidney extracts distinct enzymes act on alanine, aspartic acid, glutamic acid and phenylalanine, and suggest that a specific deaminase may exist for each amino-acid. In potato-tuber slices (Boswell, 1945) amino-acids are less specifically deaminated by quinones formed from polyphenols by polyphenol oxidase. Bacterial enzymes deaminating aspartic acid, serine and threonine require biotin for activation (Lichstein & Umbreit, 1947).

Serine breaks down in liver slices to glycine, the suggested reaction being

$$\underset{\text{Serine}}{CH_2OH.CHNH_2.COOH} = H.CHO + \underset{\text{Glycine}}{CH_2.NH_2.COOH}$$

(Leuthardt & Glasson, 1942*a*). Direct conversion to glycine of serine labelled with N^{15} in the amino-group and C^{13} in the carboxy-group was demonstrated *in vivo* for rat and guinea-pig by Shemin (1946). Serine is deaminated by *Bacterium coli* to pyruvic acid and ammonia, probably via amino-acrylic acid (Chargaff & Sprinson, 1943). Serine is also involved in the biosynthesis and breakdown of tryptophane:



(Tatum & Bonner, 1944; Umbreit *et al.* 1946). Anthranilic acid is a probable intermediate in the biosynthesis of indole (Tatum, Bonner & Beadle, 1944); it occurs, as methyl anthranilate, with indole in jasmine oil (Karrer, 1938).

Bacterium fluorescens liquefaciens has enzymes breaking down aspartic acid by the reactions

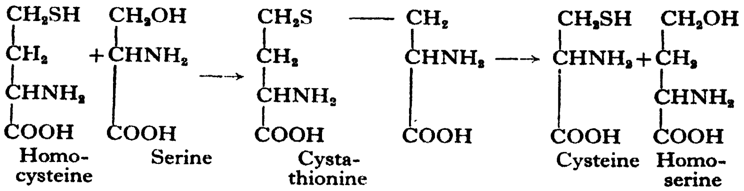
$$\underset{\text{Aspartic acid}}{HOOC.CH_2.CHNH_2.COOH} \rightleftharpoons \underset{\text{Fumaric acid}}{HOOC.CH{=}CH.COOH} + NH_3$$

$$HOOC.CH_2.CHNH_2.COOH + H_2O \rightarrow \underset{\text{Malic acid}}{HOOC.CH_2.CHOH.COOH} + NH_3$$

The latter is the first example of hydrolytic deamination of an amino-acid (Virtanen & Erkama, 1938).

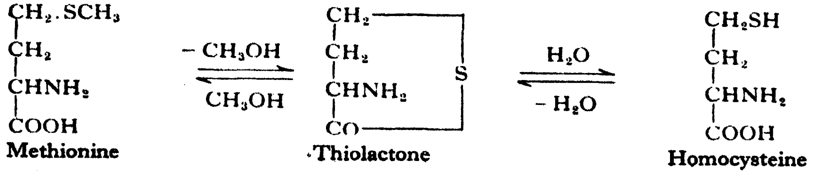
Cystine can break down in animal tissues by two routes, each finally leading to taurine (Medes & Floyd, 1942). In rat tissues Smythe (1944) found that cysteine could reversibly split off hydrogen sulphide to form amino-acrylic acid, which in turn formed pyruvic acid and ammonia. Cysteine is oxidized to cysteic acid, which can take part in transamination reactions (Bychkov, 1939; Cohen, 1940).

In the rat cysteine is formed from homocysteine and serine via cystathionine (Binkley & du Vigneaud, 1942; Stetten, 1942):



A substance, assigned the structure of cystathionine, occurs as an isomorphous compound with its selenium analogue in grain from localities with a high soil content of selenium (Horn & Jones, 1940). Reactions involved in cystine metabolism are summarized in Fig. 3.

Methionine, another sulphur-containing amino-acid, has received more attention through transfer of methyl groups in animal tissues than in connexion with nitrogen or sulphur metabolism. Simmonds & du Vigneaud (1942) demonstrated use of the S-methyl group of human dietary methionine in formation of choline and creatinine. Use of choline methyl groups in synthesis of methionine was shown (Simmonds, Cohn, Chandler & du Vigneaud, 1943) by the presence of deuteriomethyl groups in the tissues of rats fed homocystine and deuteriocholine. Betaine (du Vigneaud, Simmonds, Chandler & Cohn, 1946) is a very effective source of labile methyl groups in the animal. Syntheses by etiolated wheat seedlings of creatine from glycocyamine and betaine from glycine are increased several times by supply of methionine. Neither —SH nor —S.S— groups appear, precluding simple exchange of hydrogen for methyl on the sulphur atom. The reactions suggested (Barrenscheen & von Vályi-Nagi, 1942) are



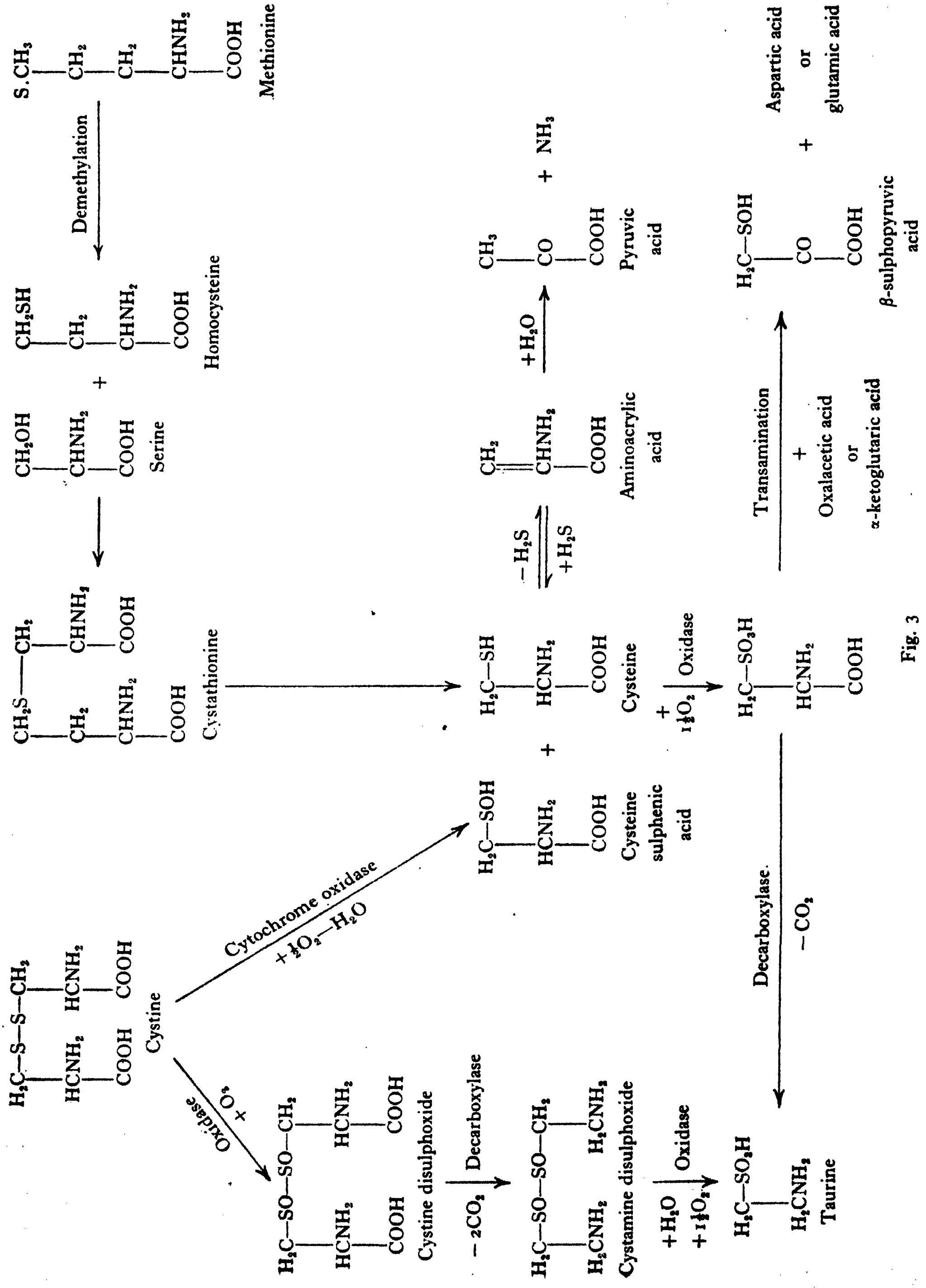
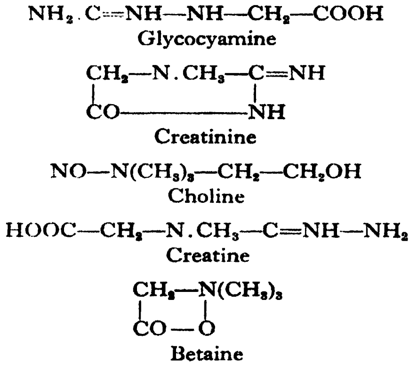

Methionine
Demethylation
Homocysteine
Serine
Cystathionine
Cystine
Cytochrome oxidase
$+\frac{1}{2}O_2 - H_2O$
Oxidase
$+O_2$
Cystine disulphoxide
$-2CO_2$
Decarboxylase
Cystamine disulphoxide
$+H_2O$
$+1\frac{1}{2}O_2$
Oxidase
Taurine
Cysteine sulphenic acid
Cysteine
$-H_2S$
$+H_2S$
Aminoacrylic acid
$+H_2O$
Pyruvic acid
$+NH_3$
$+1\frac{1}{2}O_2$
Oxidase
Decarboxylase
$-CO_2$
Transamination
Oxalacetic acid or α-ketoglutaric acid
β-sulphopyruvic acid
Aspartic acid or glutamic acid

Fig. 3

A similar formulation for reactions *in vitro* is given by Toennies & Callan (1939). Methionine also accelerates conversion of glycocyamine to creatine in liver (Borsook & Dubnoff, 1940) (for formulae see Fig. 4).



$NH_2.C{=}NH{-}NH{-}CH_2{-}COOH$

Glycocyamine

$$\begin{array}{lcl} CH_2{-}N.CH_3{-}C{=}NH \\ | \qquad\qquad\quad | \\ CO{-}{-}{-}{-}{-}{-}{-}NH \end{array}$$

Creatinine

$NO{-}N(CH_3)_3{-}CH_2{-}CH_2OH$

Choline

$HOOC{-}CH_2{-}N.CH_3{-}C{=}NH{-}NH_2$

Creatine

$$\begin{array}{l} CH_2{-}N(CH_3)_3 \\ | \qquad | \\ CO{-}O \end{array}$$

Betaine

Fig. 4

N.H. Horowitz (1947) has studied four strains of the mould *Neurospora crassa* which have lost by single-gene mutations the ability to synthesize methionine. One can use cysteine, cystathionine and homocysteine; another cystathionine and homocysteine; the third homocysteine; the fourth methionine only. Methionine synthesis is thus blocked at a different point in each strain. Cystathionine accumulates in a strain which cannot use it. The synthetic sequence

cystine → cystathionine → homocysteine → methionine

is suggested for the normal organism.

On analogous evidence the synthetic sequence

aminoethanol → monomethylaminoethanol → dimethylaminoethanol → choline

is suggested for choline synthesis in *Neurospora* (Horowitz, 1946). In the rat serine is decarboxylated to amino-ethanol (Stetten, 1942).

Choline has been largely neglected by plant biochemists in recent years. Possibly the methodological work of Street, Kenyon & Watson (1946*b*) may help to repair this omission. It has been shown by earlier workers to be widespread in plants; Street *et al.* (1946*b*) found it in all parts tested of four solanaceous species (except depleted tubers of *Solanum tuberosum*), and Müller & Armbrust (1940) in the soy bean. Betaines (especially glycine betaine and trigonelline) are other methylated compounds widespread in plants but of unknown metabolic role. It has been stated (Ackermann & Appel, 1940) that they do not occur together, but Müller & Armbrust (1940) found both in the soy bean. Betaine and choline contents remain steady in starving oat leaves (J. G. Wood & Cruickshank, 1944).

Frey-Wyssling (1938), in an interesting speculative paper, suggests that many minor plant constituents are formed by decarboxylation and deamination of amino-acids to olefines. Ethylene, important in the metabolism of ripening fruits, would thus be formed from alanine, and, by plausible further transformations, phenylethyl alcohol (found in rose oil) from phenylalanine, heteroauxine from tryptophane, diallyldisulphide (garlic oil) from methionine, and isoprene, the unit from which rubber and probably terpenes are formed, from leucine. Asparagine would give acrylic acid, readily oxidized to oxalic acid, widespread in plants but of unknown metabolic origin. Onslow (1931, 1932) suggested that anthocyans and flavones arise from deamination products of amino-acids.

PROTEINS AND THEIR SYNTHESIS

It is not proposed to consider in any detail the various controversial hypotheses of protein structure. This field has been reviewed by Bull (1941), Chibnall (1942), Astbury (1943) and Neurath, Greenstein, Putnam & Erickson (1944). Ogston (1943) analyses mathematically the implications of some of the hypotheses.

Composition of plant proteins

Vickery (1945), reviewing this field, points out that knowledge of virus proteins, whose medical significance has ensured their intensive study by the full range of modern physical technique, is much greater than for normal proteins. Vickery, Smith, Hubbell & Nolan (1941) and Smith, Greene & Bartner (1946) have studied the amino-acid composition of curcurbit seed globulins. Differences could be established between but not within genera. Lugg (1940) found that proteins of assimilating tissue of the liverwort *Lunularia cruciata*, the fern *Pteridium aquilinum* and a *Selaginella* species had tyrosine, tryptophane and amide contents similar to those of phanerogams. The liverwort protein was from the gametophyte; in the others, as with phanerogams, from the sporophyte. Lugg (1946) tabulates recent data on the amino-acid composition of viruses and of proteins from photosynthesizing tissues of thallophytes, bryophytes, pteridophytes and spermatophytes. Mazur & Clarke (1938, 1942) claim that several algae from various major groups lack amino-acids always found in other plants. Lugg (1947*b*) points out that they report very large amounts of humin nitrogen, indicating that, in all probability, some amino-acids, notably cystine, cysteine and methionine, which are among those most frequently reported absent by Mazur & Clarke, may have been largely lost to analysis.

Chibnall (1939) gives contents of nine amino-acids and of amide for vacuole, cytoplasm and chloroplast protein of spinach leaves. Protein of both cytoplasm and chloroplast has much less aspartic and glutamic acids than is found in reserve proteins of many seeds. There is a general similarity of composition between cytoplasm and chloroplast protein, but the former has more lysine and less histidine. Contents of the same nine amino-acids and of amide in the cytoplasmic protein of *Spinacia oleracea*, *Zea mays*, *Ricinus communis*, *Cochlearia armoracia*, *Crambe cordifolia* and *Phaseolus multiflorus* show a strikingly small range of variation.

Hanson (1939) states that 'Chlorophyll is a porphin compound. Free porphin compounds occur in nature only as decomposition products of the physiologically active chromoproteids.' He gives evidence that chlorophyll probably occurs in the plant in tetrads of four chlorophyll molecules on a protein carrier of molecular weight 68,000. Sideris (1947) found a chlorophyll/protein ratio approaching this value in mature pineapple leaves; young tissues had excess chlorophyll. Granick (1938*a*) reports that chloroplast nitrogen is between 30 and 40% of the total nitrogen in tomato and tobacco leaves of varying ages; synthesis of chloroplast and other protein thus keeps pace. His suggestion of independent protein synthesis in both chloroplast and cytoplasm is supported by the presence (Nezgovorov, 1941) of proteases in isolated chloroplasts.

In young leaves of Sudan grass chloroplast protein is 7% of the total but increases later to 35–40%. Total and chloroplast nitrogen and sulphur reach maxima together during the life cycle and decline later. Chloroplast proteins are rich in sulphur and have about 70% of the total protein sulphur of the leaf (Hanson, Barrien & Wood, 1941). In *Phalaris tuberosa* the protein/chloroplast ratio increases during net protein synthesis and decreases in net protein hydrolysis, suggesting surplus protein production in chloroplasts with high nitrogen supply. Loss of protein in drought and senescence is more rapid in the chloroplasts than in the rest of the cell (Hanson, 1941). Groner (1936) has shown that albino seedlings of *Zea mays* have 3–5 times as much amino-nitrogen as green seedlings of the same age and parent stock. This may be due to decreased utilization in building chloroplast protein or to a lack of carbohydrate to supply carbon for protein synthesis. Moyer & Fishman (1943) found curves of electrophoretic motility against pH for protein-chlorophyll complexes essentially similar in eleven legumes but different in *Aspidistra* (Liliaceae).

Relations between nitrogen and sulphur in the protein metabolism of *Lolium multiflorum*, *L. subulatum* and *Andropogon sudanense* suggest that with increased nitrogen supply more sulphur is used in protein formation, but leaf protein is concurrently diluted with a low-sulphur, more readily utilizable component (J. G. Wood & Barrien, 1939*a*, *b*; Barrien & J. G. Wood, 1939). Sulphur-deficient plants show reduced utilization of nitrate, and, in spite of high carbohydrate contents, marked protein hydrolysis (Eaton, 1941).

Protein synthesis

(*a*) *General*

The usual assumption that protein synthesis is essentially a reversal of its hydrolysis has been challenged by Alcock (1936), who suggests initial formation, possibly by condensation of formaldehyde with ammonia or nitrite, of a simple unit whose polymerization and internal differentiation form protein. Proteins typical of individual species arise from a precursor common to all organisms. Hall (1937) derives amino-acids for protein synthesis from 2-aminohexoses. These compounds occur in fungi (van Iterson, Meyer & Lotmar, 1936) and green plants (Sideris *et al.* 1938*b*), but are best known as monomers of complex animal structural materials, e.g. chitin, and of some animal products of physiological importance (Haworth, 1947). There seems, however, no evidence demanding abandonment of the amino-acid hypothesis of protein formation. An alternative route is possible, as observed utilization of amino-acids in protein formation can always be interpreted as due to transfer of their nitrogen to some other precursor. Alternative suggestions, though less precise than reversal of hydrolysis, should be kept in mind in case the latter hypothesis becomes untenable. Beadle (1945) produced by irradiation mutants of the fungus *Neurospora* biochemically defective in needing amino-acids not essential to the normal type. Ability to synthesize each of eight amino-acids seems a unit character, suggesting they are formed separately and required individually in the normal type. Formation of protein from simple units and subsequent differentiation into amino-acid residues is thus improbable here.

There is *a priori* no reason to assume an identical mode of synthesis for all proteins. Genes, viruses and some enzymes are proteins formed autocatalytically. Several workers, e.g. Gulick (1938), suggest that an existing protein molecule copies itself by attracting simpler molecules (e.g. amino-acids) with structures similar to its component parts. Adsorption of attracted molecules by portions of the protein molecule whose structure is like their own ensures their arrangement in the right pattern to copy it. Gulick (1938) writes of genes: 'In addition to the autocatalytic effect they exert as their second activity an enzyme-like control over the formation of active substances that gives them a sort of long-distance control over cytoplasmic happenings.' The analogy between genes and those sparsely distributed enzymes of which one or a few molecules occur in each cell is stressed also by McIlwain (1946*b*). As Petrie (1943) states, autocatalytic synthesis, though plausible for gene and virus proteins, is less likely for cytoplasmic and storage proteins. Spiegelman & Kamen (1946) suggest that 'genes *continually* produce at different rates partial replicas of themselves which enter the cytoplasm. These replicas are nucleoprotein in nature and possess to varying degrees the capacity for self-duplication. Their presence in the cytoplasm controls the type and amount of proteins and enzymes synthesized.'

This autocatalytic property is probably the fundamental attribute of living matter. It has at times been held that the first organisms must have been autotrophic and capable in particular of making carbon dioxide their carbon source. Oparin (1938), however, marshals much astrophysical and geochemical evidence that the original terrestrial atmosphere had methane and other hydrocarbons, ammonia and water vapour but no oxygen, nitrogen or carbon dioxide. He suggests that organic compounds, in the absence of micro-organisms to destroy them, formed slowly from ammonia and hydrocarbons in the primary sea. An autocatalytic complex molecule developing in such a sea would have a supply of simpler molecules for building units in its own reproduction. Absence now of such semi-organisms does not invalidate the hypothesis, for both they and their substrates would if arising *de novo* be destroyed by the present more advanced micro-organisms. Horowitz (1945) extends these speculations by pointing out that multiplication of such semi-organisms would eventually stop through depletion of the substrate. Then 'by a process of mutation a means was eventually discovered for utilizing other available substances. With this event the evolution of biosyntheses began. The conditions necessary for the operation of the mechanism ceased with the ultimate destruction of the organic environment. Further evolution was probably based on the chance combination of genes, resulting to a large extent in the development of short reaction chains utilizing substances whose synthesis had been previously acquired.'

Francis (1925) points out that rusting iron absorbs water, carbon dioxide and ammonia from the atmosphere and can form compounds with the other elements occurring in protein. He suggests that iron may have acted as an assembling agent in the primary formation of protoplasm and claims (Francis, 1932, 1933, 1934, 1935) to have demonstrated protein formation by sterile inorganic material. A wire of pure iron sterilized by heating to redness was suspended in a solution containing ammonium, nitrate, potassium, magnesium, phosphate, calcium, chloride and sulphate. Containers and solution were sterilized by steaming for half an hour on 3 successive days. It seems probable that this standard sterilization procedure would destroy any iron bacteria; thermophily is reported only for two species doubtfully referred to this group (Cholodny, 1926). A film, largely composed of ferric hydrate, formed on the wire at the air/solution interface and on microscopic examination after 7–14 days showed minute bodies giving ten microchemical tests for protein. Presence of atmospheric carbon dioxide was essential for their formation, none being found in its absence. Sixty preparations were examined microscopically without any indication of organisms being found. It may be mentioned that the author had studied iron bacteria before this work, and that the bodies he reports show no resemblance either to any known iron bacteria or to the chromatin granules found in the protoplasm of the iron bacterium *Leptothrix ochracea.* No repetition of this striking work, which may not be widely known, seems to have been published.

(*b*) *Chemical aspects*

Plasteins. Synthesis ot complex products from protein hydrolysates by pepsin was reported by Danilevski (1886) and Mikhailov (1886). Subsequent work has failed to elucidate completely the relation of these products to true protein. Lavrov (1907) showed that plasteins contain sulphur but rather less nitrogen than most proteins. Henriques & Gjaldbäk (1911) found that the synthetic products could have as few formol-titratable (free amino) groups as true proteins. Menzorov (1939) showed that purified pepsin but not peptidases hydrolysed plastein formed by pepsin from egg albumen digests. Collier (1940) reports that crystalline papain acts on proteose from egg albumen to give a material similar to true protein, with few free amino, carboxy or tyrosine groups and precipitable by trichloracetic acid. Synthetic activity seems established, but the relation of the product to the protein originally hydrolysed is still uncertain. A reaction forming products of doubtful composition from equally ill-defined substrates is hardly suitable for studying the mechanism of synthesis. Studies of synthesis of peptide bonds between simple compounds of known structure will probably clarify protein synthesis more than work on plasteins. The earlier literature on plasteins has been reviewed by Wasteneys & Borsook (1930).

Synthesis of peptide bonds. Bergmann & Fraenkel-Conrat (1938) synthesized a peptide bond between benzoyl-*l*-leucine and *l*-leucine anilide, using activated papain. Chymotrypsin, at a temperature and pH optimal for its hydrolytic action, can also synthesize benzoyl-*l*-tyrosylglycine from benzoyl-*l*-tryosine and glycine anilide (Bergmann & Fruton, 1938). The low solubility of these peptides favours synthesis. This work is discussed and extended by Bergmann & Fruton (1944), who consider the possibility that synthesis and hydrolysis *in vivo* of peptide bonds may be coupled with one another so that digestion of a protein by a mixture of proteolytic enzymes may not be only a sequence of hydrolytic reactions. Coupling of protein synthesis with phosphorylation and oxidation-reduction processes is also considered and has been supported by more recent work. Cohen (1945) stresses that thermodynamical equilibrium is almost completely in favour of hydrolysis of peptide bonds, unless special conditions are invoked, and suggests synthesis may not be a mere reversal of hydrolysis. He cites other syntheses *in vivo* known to be coupled with oxidation-reduction systems via energy-rich phosphorylated intermediates, and mentions the possible condensation of phosphorylated amino-acids, or of phosphorylated α-keto-acids with amino-acids, to give ketopeptides. A similar hypothesis of biological peptide synthesis by acylation and amination of α-keto-acids is due to Herbst & Shemin (1943), who synthesized alanylalanine from pyruvylalanine in a model system with α-aminophenylacetic acid as amino-group donor. Spiegelman & Kamen (1946) state that in yeast protein synthesis, with liberal supplies of nitrogen or on transfer to media requiring new enzymes, is associated with release of phosphate from nucleoprotein, and suggest formation of energy-rich phosphorylated intermediates. Peptide bonds once formed, the building of protein structure seems not to involve excessive energy requirements. Butler (1946), by calculations 'admittedly rough and based on inadequate data', concludes that configurational entropy is not a dominant factor in the free energy of protein. Complete oxidation of a glucose molecule should give enough energy to build to protein about 100 amino-acid residues.

Bovarnick (1942) investigated a polypeptide secreted into its culture medium by *Bacillus subtilis*. It is particularly suitable for the study of synthesis, being available outside the cell and composed of residues of glutamic acid only. Deuterium labelling showed α-iminoglutaric acid or a derivative is involved in its synthesis. He suggests formation of the peptide bond by a simple keto-acid or aldehyde with subsequent metathesis with glutamic acid is more likely than condensation with α-keto-δ-aminoadipic acid; in general, this process 'eliminates the necessity for postulating a strange variety of α-keto-acids', and is analogous with such known metathetical reactions as formation of glycogen from glucose monophosphate. Cohen & McGilvery (1947*a*, *b*) demonstrated formation of *p*-aminohippuric acid by rat-liver homogenates from *p*-aminobenzoic acid and glycine. The reaction is stimulated by succinate, fumarate, malate, oxalacetate, citrate, α-ketoglutarate, glutamate and pyruvate, all members of or closely related to the tricarboxylic acid cycle, and requires adenosine triphosphate. The authors conclude that 'a mechanism is indicated in which synthesis occurs upon transfer of high-energy phosphate, possibly to one of the reactants'. Kuzin & Polyakova (1945) found a marked stimulation by hexoses of synthesis of benzoyl-glycine-anilide and benzoyl-glycine-anilide by papain; the mechanism of the stimulation is not clear.

The recent synthesis by Woodward & Schramm (1947) of protein analogues of very high molecular weight may also lead eventually to a better understanding of synthesis *in vivo*. Anhydrides of *N*-carboxy-α-amino-acids are polymerized in a non-aqueous medium in a reaction initiated by water or any substance with an active hydrogen atom. Each time a new peptide bond is formed an active centre is regenerated by spontaneous loss of carbon dioxide from the unstable grouping (—NH.COOH). The products of the chain reaction have molecular weights from 2 to 15 million.

Regulation of protein synthesis and breakdown

In plants (Rittenberg *et al.* 1939; Hevesy, Linderstrøm-Lang, Keston & Olsen, 1940) as in animals (Foster *et al.* 1939; Schoenheimer, Ratner, Rittenberg & Heidelberger, 1942; Shemin & Rittenberg, 1944) isotope techniques have demonstrated continuous interchange between the nitrogen of tissue constituents and that supplied externally. The protein content of tissues, which may in the absence of excessive environmental fluctuations appear fairly steady, is thus the resultant of continuous opposed processes of synthesis and breakdown. Moreover, ammonia, amino-acids and protein in tissues are far from being in equilibrium with each other (Borsook & Dubnoff, 1943). Energy, derived ultimately from oxidation, must be consumed in maintaining as well as synthesizing protein in the plant.

Regulation of protein metabolism in plants has been much studied without a clear picture of its mechanism emerging. The field has been reviewed by Petrie (1943) and J. G. Wood (1945). Chibnall (1939) has suggested that protein level in leaves is subject to hormonal control, and Ali-Zade (1941) (see p. 15) reports results most readily interpretable in such terms. Kursanov & Bryushkova (1940), using the technique described by Kursanov (1941), infiltrated amino-acid mixtures into leaves of wheat at varying stages of development and found that at the time of inflorescence formation all leaves simultaneously lost the power of synthesizing protein and hydrolysed stored protein, which was translocated to the growing reproductive organs. Later the leaves regained their synthetic activity. Pearsall & Billimoria (1938) also state that leaf protein is low at the time of flowering. Rubin (1945) quotes unpublished work by Artiskhovskaya showing a greater rate and extent of protein breakdown in a poor-keeping compared with a good-keeping cabbage variety.

Attached leaves of barley synthesize protein less effectively with increasing age, but mature leaves retain synthetic ability (Walkley, 1940; Walkley & Petrie, 1941). Pearsall & Billimoria (1938, 1939) found that mature leaves of several species floated on nutrient solutions with glucose and ammonium nitrate had little capacity for protein synthesis. Young *Pelargonium* leaves synthesized more protein from asparagine than from ammonium nitrate. The elongating bases of *Narcissus* leaves could synthesize protein. Disks from mature cotton leaves synthesize protein from nitrate as effectively as leaves attached to the plant (Phillis & Mason, 1942). Steward, Berry, Preston & Ramamurti (1943) showed that protein decreases, amino-acids accumulate and capacity to synthesize protein is progressively lost in potato tubers stored at 2° C. but not at 11° C. Levitt (1946) found considerable protein synthesis in potato tubers after transfer from 0 to 25° C.

There is thus in some cases evidence for hormonal control, and in others for protein synthesis in isolated organs. Protein regulation in the leaf and other parts of the plant seems to involve both local and general factors, as well as the effects of translocation from mature tissues to regions of active growth.

Relations between protein and other plant constituents

(*a*) *Carbohydrate.* Paech (see McKee, 1937) suggested that protein content is controlled by the contents of ammonia and hexose. His work is criticized by Chibnall (1939), who stresses the essential weakness of the assumption that amino-acid content, and so protein content, is determined by hexose content. The immediate precursors of amino-acids are α-keto-acids, which need not be in equilibrium with hexoses. The hypothesis was experimentally tested by J. G. Wood & Petrie (1942), who supplied varying amounts of sucrose and ammonium to *Phalaris tuberosa* plants. Protein showed no definite relation to either ammonium or carbohydrate, but 90% of the variance of the protein content could be accounted for in terms of amino-acid and water contents. Amos & J. G. Wood (1939) supplied rye grass (*Lolium subulatum*) with varying amounts of ammonium, and analysed leaves after the plants had reached a steady state in a constant environment in light. Sucrose, glucose, fructosans and starch were all used in protein synthesis, but not fructopyranose. Kabos (1936), supplying *Sinapis alba* seedlings with glucose, found no regular relation between carbohydrate and protein. Plants supplied with glucose maintained their protein content in the dark, in contrast to those without added carbohydrate. Protein synthesis increased with the carbohydrate supply up to 2%, but was not further increased at 6%. He suggests that relations between carbohydrate and protein can be explained by preferential respiration of carbohydrate when present. This is in agreement with observations of J. G. Wood *et al.* (1943) and Vickery & Pucher (1939) that in detached leaves high carbohydrate content delays protein hydrolysis. Postma (1939) showed that protein synthesis from nitrate in isolated roots requires added glucose.

(*b*) *Amino-acids.* Petrie & J. G. Wood (1938*a*, *b*) found a well-marked relation, expressed by a curve concave to the amino-acid axis, between protein and amino-acid content in leaves of the grasses *Phalaris tuberosa* and *Lolium multiflorum*. They suggested the concavity of the curve relating protein to amino-acid content might be due to differential rates of increase of individual amino-acids, and showed cystine was, in their experiments, out of step with the other amino-acids. The form of the curve was not altered by deducting the amino-groups of amides from the total amino-acids. The same general relation is reported for other types of material (Walkley & Petrie, 1941; J. G. Wood & Petrie, 1942; J. G. Wood, 1942).

J. G. Wood and his co-workers have made a technical advance in the study of starving leaves by determining individual amino-acids as well as total amino-nitrogen. They have shown (J. G. Wood *et al.* 1943, 1944; J. G. Wood & Cruickshank, 1944; J. G. Wood & Womersley, 1946) that carbohydrate is lost in nitrogen at the same rate as in air but protein is not metabolized. Chloroplast protein, ascorbic acid and chlorophyll decrease in air at similar rates, but are all stable for long periods in nitrogen. J. G. Wood & Cruickshank (1944) suggest that protein normally exists in the chloroplast as a complex, inaccessible to proteolytic enzymes, with chlorophyll, carotenoids, phosphatides and ascorbic acid, similar complexes occurring in the cytoplasm also. Weier (1938) reviews evidence for such association in the chloroplast and Przyłecki (1939), the types of complex known to be formed by protein. It has been shown (Fagan & Ashton, 1938; Smith & Wang, 1941; Smith & Robb, 1943; Keirstead, 1945) that in several species nitrogen and carotene contents of leaves from plants at different stages of development and with varied fertilizer treatments are highly correlated. Fluctuations of chlorophyll and carotene contents of developing rice, barley and wheat plants follow similar courses (Nagasima, 1938). Chlorophyll and carotene are correlated with nitrogen level and so, presumably, with protein in pineapple leaves (Sideris & Young, 1947). These workers, however, and Wynd (1946) with cereal leaves find more complex relations between protein and ascorbic acid. The leaf concentration of ascorbic acid seems to vary more between species than is the case with chlorophyll, carotene and protein; in leaves where its concentration is high, ascorbic acid may exist only in part as a complex with protein.

Such a complex is considered to be broken down in detached leaves in air by oxidation of its components, which would in the intact plant be replaced by synthesis. Injured leaves in nitrogen metabolize protein to amino-acids but do not synthesize amides (J. G. Wood & Cruickshank, 1944). These workers also found differential oxidation of amino-acids in kikuyu grass and oats starved in air; the order of utilization of those estimated was cystine, glutamic acid, arginine, tyrosine and tryptophane. In starving oat leaves in air there is no change in betaine, choline or purines.

These new methods of attack on metabolic problems in starving leaves allow a more detailed formulation of the probable sequence of events. J. G. Wood *et al.* (1943) make the following suggestions for kikuyu grass and Sudan grass: (1) One or more amino-acids, including cystine, are oxidized to ammonia and other compounds at a rate depending on the sucrose content. (2) Sulphur-rich protein, including chloroplast protein, is hydrolysed to restore equilibrium among the amino-acids. Hydrolysis of chloroplast protein is accompanied by loss of chlorophyll and ascorbic acid. (3) Glutamine is formed from ammonia (from oxidation of amino-acids) and glutamic acid (from protein hydrolysis), and also from ammonia and α-ketoglutaric acid. (4) Asparagine is formed from ammonia and aspartic acid (from protein hydrolysis). (5) Citric acid is formed from pyruvic acid (from glycolysis) and oxalacetic acid or malic acid at a rate determined by the contents of sucrose and oxalacetic acid. (6) Oxalacetic acid is formed from aspartic acid or by oxidation of citric acid or α-ketoglutaric acid (from glutamic acid). Malic acid is produced in equilibrium with oxalacetic acid. With a falling respiration rate α-ketoglutaric acid increases at the expense of glutamic acid. Malic acid and oxalacetic acid increase by oxidation of α-ketoglutaric acid, and aspartic acid, formed from oxalacetic acid and amino-acids by transamination, combines with ammonia to form asparagine.

Lugg & Weller (1941) showed that 90% of the methionine of the embryo of *Lupinus angustifolius*, compared with about 30% for tyrosine, tryptophane and cystine plus cysteine, could be accounted for in the seedling protein. They suggest that methionine supply may limit protein regeneration in the seedling. The correlation (Strebeyko, 1939) between production of insoluble phosphorus and insoluble nitrogen in barley seedlings may indicate a similar effect with phosphorus compounds. Williams (1938, 1946) showed that soluble nitrogen accumulates in phosphorus deficiency and phosphorus is directly concerned with protein synthesis in *Phalaris tuberosa*. The rate of protein synthesis in tobacco increases with phosphorus supply (Watson & Petrie, 1940).

Relation of proteins and amino-acids to respiration

Gregory & Sen (1937) found high correlations between respiration and both protein and amino-acid contents in barley leaves. Petrie (1943) points out that, as their data were expressed per unit dry weight, correlations between protein and respiration may indicate only that protein is a rough measure of respiring protoplasm. Petrie & Williams (1938) also concluded that correlation between protein and respiration rate was due to this effect. Pears picked at different maturities show considerable differences in protein content and in initial respiration rate, but their ratio remains steady (Kidd, West, Griffiths & Potter, 1940). There seems to be a phase of net protein synthesis, associated with the climacteric of respiration, during storage, as in apples (Hulme, 1936) but not plums (Donen, 1937, 1938). Hulme (1946) suggests that much of the protein he has succeeded in extracting in soluble form from the acid tissues of the apple is enzymatic in nature. This might mean the respiratory climacteric is associated with an increased enzyme content in the cells; it should be remembered, however, that a similar increase in respiratory activity is found in detached leaves with continuous protein hydrolysis. Changes in the ethylene content of fruits might affect their protein metabolism; it is stated (Turkova, 1945) to inhibit proteases at low but to stimulate them at higher concentrations.

Steward & Preston (1940, 1941 *a, b*) and Steward, Stout & Preston (1940) consider that respiration, salt accumulation and protein synthesis in potato disks are interrelated. They separate two components of respiration. One, independent of protein synthesis and salt uptake, provides one-third of the respiration in distilled water. The other provides two-thirds of the basal respiration and increases during protein synthesis and salt uptake. Boswell & Whiting (1938) also recognize two components, in the ratio 1 : 2, of the respiration of potato disks.

Non-amide amino-acids appear important respiratory substrates in the early development of barley seedlings (Brown, 1946). This may be compared with the stimulation by amino-acids of oxygen consumption by submerged plants of *Elodea canadensis* (Schwabe, 1932). In *Lemna* (White & Templeman, 1937), tomato and wheat (Hamner, 1936) respiration is positively correlated with nitrogen supply. Decrease in dry weight with increased nitrogen supply in early growth of wheat and Sudan grass (Ballard & Petrie,

1936) may be due to stimulation of respiration. Shcherbakov (1945) states that total weight loss in etiolated pea seedlings deprived of potassium much exceeds carbohydrate loss; he concludes (without confirmatory analyses of nitrogen fractions) that in potassium deficiency the respiratory substrate is largely protein. Protein hydrolysis with accumulation of amino-acids occurs in potassium deficiency (Alten, Goeze & Fischer, 1937; Hartt, 1934; Gregory & Sen, 1937; Nightingale, 1937*b*).

In brain tissue glutamic acid inhibits anaerobic glycolysis in bicarbonate but stimulates it in phosphate buffer or in aerobic conditions. The effects are not accompanied by measurable transamination (Grodzenski, 1940; cf. Weil-Malherbe, 1938). The different effects in the two buffers show that experiments in which they are used must be interpreted with caution, as both phosphate and bicarbonate are metabolically active.

Respiration rate is largely independent of carbohydrate content when this is high. J. G. Wood (1942, 1945) points out that this implies not a real lack of effect of carbohydrate on respiration but merely that the effect reaches a maximum. At high carbohydrate contents the correlation between respiration and amino-acid content becomes more apparent.

The initial respiration rates of leaves of barley plants at varied levels of phosphorus and potassium supply show, when adjusted for the level of other factors, a close and simple relation with protein content (Richards, 1938). Over a wide range of nitrogen, phosphorus and potassium supply the lowest respiration rate, per unit protein, is in plants with a full-balanced nutrient supply. He suggests that a definite rate of carbon dioxide production is required to maintain a given amount of protein. In starving leaves, however, respiration rises, after an initial fall, to a well-marked maximum, while protein is steadily hydrolysed (Yemm, 1937; J. G. Wood *et al.* 1943, 1944). J. G. Wood & Cruickshank (1944) modify the hypothesis by suggesting that the rate at which an oxidative cycle operates on carbohydrate may determine protein content in leaves. Maintenance of protein content depends on synthesis of the most readily oxidized amino-acids keeping pace with their oxidation. Rate of carbohydrate oxidation could control this synthesis either through linked energy-providing reactions (probably involving high-energy phosphate bonds), or through production of essential non-nitrogenous intermediates (probably oxalacetic and α-ketoglutaric acids).

Effect of water content on nitrogen metabolism

Decreasing water content in leaves causes protein hydrolysis (Petrie & J. G. Wood, 1938*a*, *b*). Relations of ammonia to amino-acids and amides are, however, largely independent of the water content of the tissue (J. G. Wood & Petrie, 1938). Petrie (1943) attributes, at least in part, the early hydrolysis of protein found by Yemm (1937) in detached barley leaves, to a falling water content during the experiment. Yemm (1947) stresses, however, that the water content of the leaves has since been shown to be constant in his conditions.

Effects of mineral elements on nitrogen metabolism

The roles of sulphur and phosphorus (pp. 37, 43) and of potassium have been considered. Bennett (1945) associates chlorosis in iron deficiency with a deep disturbance of nitrogen metabolism. In severely chlorotic pear leaves total nitrogen is about the same as in green leaves, but soluble nitrogen is five times as great. Application of iron causes greening of the leaves and restores the normal ratio of soluble and insoluble nitrogen. Iron deficiency thus seems to impede formation of protein from soluble precursors. Guest (1943) quotes similar results with leaves of *Macadamia ternifolia* var. *integrifolia* (Proteaceae). Copper-deficient tung trees (*Aleurites fordii*) (Euphorbiaceae) show markedly increased deposition of insoluble nitrogenous compounds in leaves and fruit hulls (Gilbert, Sell & Drosdoff, 1946). Excess chloride in the soil decreases the protein/non-protein ratio in *Pelargonium* (Schneider, 1935) and produces an accumulation of ammonia in the leaves of cotton (Strogonov & Ostapenko, 1946).

Protein content of grains as affected by climatic conditions

In wheat, and to a lesser extent in other cereals, commercial value increases with protein content; factors influencing the latter have therefore received much study. The final protein content of grain results of course from the balance of the rates of formation of protein and of other constituents, but published work rarely permits separation of such processes. McGinnis & Taylor (1923) concluded that differential rates of carbohydrate respiration could account for only a comparatively small portion of the variation in protein content in wheat of the same variety grown in different localities. Such variation can be very large, for example McGinnis & Taylor got with Marquis wheat 9·2% protein at Puyallup, Washington, and 18·2% at Havre, Montana; oats and barley gave similar results but with a smaller range of protein content. Le Clerc (1910) got 11·2% protein in Crimean wheat grown in California and 22·4% in Kansas, and Breakwell & Hutton (1939) 6·8–19·0% in Ford wheat grown in different localities in South Australia.

Climatic variations seem the main factor determining protein content. Ivanov (1926) grew five varieties of wheat at two Russian localities of almost the same longitude but separated by 9° 23′ of latitude. Protein content at the southern locality averaged 17·3% and at the northern 11·4%. The effect of locality was greater with some varieties than others, as observed also in Portugal by Pereira (1944), but Knyaginichev (1939) states that variation between varieties in any one year is much greater than between their long-term averages. Ivanov & Knyaginichev (1936) give maps summarizing very extensive data on the protein content of wheat, oats and barley in all cereal-growing areas of U.S.S.R. The maps for all three cereals are very similar, showing high protein contents in areas of hot, dry summers, as was found also in South Australia by Breakwell & Hutton (1939). Paull & Anderson (1942) show that in Canadian prairie conditions protein content is inversely correlated with rainfall during the growing period. The large seasonal variations observed in the average protein content of wheat in Victoria (Australia) (O'Brien, 1946) also emphasize the importance of climatic factors. Mangels (1925) points out that in North Dakota years when protein content is low are also years of high yield, and so produce more protein per acre.

Soil nitrogen also affects the protein content of grain. Mangels (1925) states that wheat following legume crops has a high protein content, and Helgeson & Harris (1941) showed that the protein content of Thatcher wheat in water culture could range from 11·1 to 24·9% with varying nutrient supplies.

The proportion of gluten, important in bread-making, to total wheat protein is also largely determined by climatic effects (Scharnagel & Aufhammer, 1942; Pereira, 1944).

Australian wheat is grown in areas with, in most years, hot, dry summers; its characteristically low protein content seems therefore anomalous. It may be due to the system of wheat marketing which, unlike those of other major wheat-exporting countries, sets no premium on high-protein wheat. Use of varieties of inherently low protein content is thus encouraged if they have satisfactory general agronomic qualities.

Clements (1940), studying sugar cane in localities on Oahu (Hawaii) differing little in temperature but greatly in average light intensity, concluded that the nitrogen requirement of the crop is determined primarily by the climate. Plants grown in cloudy localities had about 2·5 times as much nitrogen, per unit dry weight, in the stems as those from sunny areas, yield of sugar per acre being in the inverse ratio. Nitrogen content of the more active plant parts (leaves, meristems) was almost independent of locality. Nightingale (1942*a*), also in Hawaii, showed that the nitrate requirement of pineapples varied markedly from year to year, owing to varying climatic conditions.

ALKALOIDS

General

Published work on the alkaloids has been extensive and varied in outlook. Scientific interest was first drawn to these compounds by their remarkable effects on the animal organism and later by the fascination for organic chemists of working out their structures, which, though relatively complex, were mainly accessible, unlike those of the proteins, to the methods of classical organic chemistry. The elucidation of alkaloid structural chemistry is still incomplete, but is now largely concerned with the study of cases of particular difficulty, such as strychnine, or of alkaloids from species not previously examined chemically. In recent years the emphasis of interest has shifted from alkaloid structure to their place in plant metabolism, and the biochemical reactions involved in their synthesis and breakdown. There has recently been much work on *ad hoc* problems in alkaloid production, but the output of more fundamental papers has also been considerable.

Distribution among plant species

McNair (1941*a*, *b*, 1943), considering the taxonomic distribution of alkaloidal genera and species, concludes that plant types, e.g. trees, epiphytes, hydrophytes and total parasites, whose saps have typically low concentrations of electrolytes are much less likely to produce alkaloids than those, e.g. herbs, geophytes, xerophytes and halophytes, with more concentrated saps. Similar conclusions are advanced for organic sulphides and cyanogenetic glucosides.

Many groups of structurally related alkaloids are known only from groups of related species, or even from a single species, but as more species are examined some alkaloids supposedly confined to close taxonomic groups have been found in widely separated species. *Anabasis aphylla* (Chenopodiaceae) contains lupinine—otherwise known only from Papilionatae and one species each of Berberidaceae and Papaveraceae—and anabasine, found also in *Nicotiana* species (Orekhov & Menshikov, 1931; Henry, 1939). The tropane alkaloids are characteristic of certain members of the Solanaceae and Erythroxylaceae, but convolvine and convolvamine from *Convolvulus pseudocantabricus* belong (Orekhov & Konovalova, 1934, 1935) to this group. Salsoline from *Salsola richteri* (Chenopodiaceae) is closely related to carnegine from *Carnegia gigantea* (Cactaceae) (Späth, 1929; Orekhov & Proskurnina, 1934). Nicotine was long known only from *Nicotiana* species but has now been found in *Lycopodium complanatum*, *Equisetum arvense* (Manske & Marion, 1942) and

Asclepias syriaca (Marion, 1939). *Duboisia myoporoides* (Solanaceae) has two minor alkaloids, ψ-tropine and dihydroxytropane, both known elsewhere only from *Erythroxylon coca* (Barger, Martin & Mitchell, 1937).

These instances show that species widely separated systematically can form alkaloids once thought specific for certain taxonomic groups, yet in doubtful cases a combination of chemical data on alkaloid structure and anatomical data on morphological structure may be useful in systematics (Manske, 1944).

Function in plants

The function of alkaloids in the plants which produce them has occasioned discussion, based largely on teleological arguments, ever since their existence has been known. They have been variously regarded as a means of protection against attack by animals, insects or fungi, as a nitrogenous reserve, as end-products of a detoxification mechanism, their deposition in dead tissues being seen as analogous to excretion in animals, and as more or less fortuitous by-products of nitrogen metabolism. They may be, however, physiologically as well as chemically a heterogeneous and possibly artificial group. Certainly evidence can be produced against any generalized view of their functions put forward so far. Nicotine and anabasine, though important insecticides, are not toxic to all types of insect, and insect damage to crops, such as tobacco, tomato and potato, whose leaves contain alkaloids, is well known. Crops grown solely for their alkaloid content, e.g. *Hyoscyamus* (Barnard & Finnemore, 1945) and *Strychnos nux-vomica* (Sabalitschka & Jungermann, 1926), have been said to suffer from severe insect attack. It is, nevertheless, possible that alkaloidal plants may gain a real, though only occasional, selective advantage at times of severe infestation by insects sensitive to their alkaloids. B. Horowitz (1947) reports, however, that several insects, normally controlled by nicotine, attack *Nicotiana rustica* in spite of its particularly high nicotine content. Resistant races appear to develop. Some alkaloidal plants, e.g. *Datura stramonium*, become prominent in over-grazed pastures, but thorny plants appear even more successful in these conditions. Chatterjee (1943), studying the distribution of berberine in eight *Berberis* species, considered deposition of this very bitter substance in the outer bark a protection against animal attack. Certain species resistant to the root-rot fungus *Phymatotrichum omnivorum* contain (Greathouse & Watkins, 1938; Greathouse, 1939) in their roots and rhizomes alkaloids (berberine in *Mahonia trifoliata* and *M. swaseyi* of the Berberidaceae and sanguinarine in *Sanguinaria canadensis* of the Papaveraceae) which in culture inhibit in very low concentrations the growth of this fungus. Fischer & Gäumann (1929) state, however, that *Botrytis cinerea* is very insensitive to alkaloids, growing normally in a 4% solution of quinine sulphate.

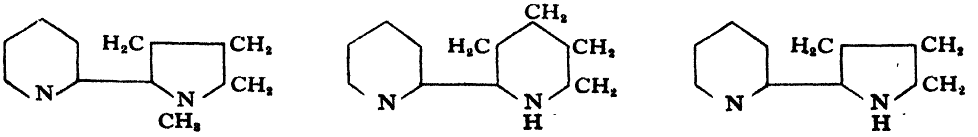
Alkaloids seem unlikely to act as a reserve of nitrogen; their N/C ratio is low and they are mostly deposited in small amounts. They could more plausibly be seen as reserves of preformed heterocyclic rings required in the formation of substances (e.g. coenzymes) essential to the metabolism of the plant, but these are synthesized with apparent ease by non-alkaloidal plants.

There is evidence that some alkaloids enter into the general metabolism of the plant. Wallebroek (1940) reported an association between alkaloid and amino-acid metabolism in seedlings of *Lupinus luteus* and considered that sparteine and lupinine play an active part in the changes during germination. They are mainly synthesized in regions of active growth, as found with solanaceous species by James (1946*a*), who showed that alkaloids are formed inside incipient vacuoles and held later in the vacuoles of a storage tissue. Nicotine is synthesized in tobacco during its period of growth, reaches its maximum content per plant at flowering and decreases considerably thereafter (Vladescu, 1934). Sabalitschka & Jungermann (1926) found considerable changes in the absolute alkaloid content of germinating seedlings of *Strychnos nux-vomica*, a species whose seeds are rich in alkaloids, and James (1946*b*) showed that the embryos and endosperm of resting seeds of *Atropa belladonna* and *Datura stramonium* are free of alkaloids, which appear very early in germination. Young leaves actively synthesize alkaloids. They break down in senescent leaves, but detached young leaves in the dark, catabolizing protein, may show alkaloid synthesis, which is increased by supplying ammonium sulphate plus sucrose. In its earlier stages of development *Papaver somniferum* has morphine in the leaves and roots, but in the mature plant the capsule, with the upper part of the stem, contains all the alkaloid (Hills, 1945). Bucherer & Enders (1942) show that nicotine is broken down by some bacteria to ammonia. Fodor & Reifenberg (1927) and Enders & Glawe (1942) provide evidence that tobacco leaves contain enzymes capable of breaking down nicotine. Dawson (1940) found that excised tobacco shoots metabolize part of their nicotine and reports (Dawson, 1946) that roots of tobacco plants supplied with extra nicotine in sand culture absorb much more nitrate than controls, metabolizing it to ammonia and amides. Solanine is found in much higher concentrations in potato sprouts, especially if sprouting in light, than in the tubers; it is present in particularly high concentrations in the flowers and is apparently used up in the metabolism of the older leaves (Naumov, 1938; Arutyunyan, 1940; Lampitt, Bushill, Rooke & Jackson, 1943; Wolf & Duggar, 1946).

In contrast with these examples of alkaloids taking part actively in the metabolism of the plant, they appear in other species, e.g. *Berberis* (Chatterjee, 1943), to be stored mainly in dead cells and, once formed, to take no further part in metabolism. Experiments, described later, in which grafted plants are grown without their normal alkaloids or with alkaloids foreign to them show that the nature of the alkaloid can be changed without greatly affecting the life of the plant.

Biogenesis

(*a*) *Studies on hybrids.* The biogenesis of the alkaloids of *Nicotiana* species, their distribution between the species of the genus, in grafted plants and in the progeny of interspecific hybrids, have been extensively studied. Frankenburg (1946) discusses much of the work. Three main alkaloids are involved, nicotine, anabasine and nornicotine. (A nor-alkaloid differs from the parent compound for which it is named in the absence of a methyl group.) Their formulae are shown in Fig. 5. Shmuk & Borozdina (1941) examined the alkaloids of forty-four *Nicotiana* species, of which eighteen, all of American



Nicotine Anabasine Nornicotine

Fig. 5

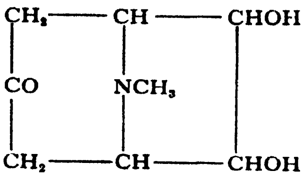
origin, had nicotine without appreciable admixture of other alkaloids, fourteen, from America and Australia, had nornicotine, nine (eight American and one Australian) had a mixture of nicotine and nornicotine, two Australian species had mixed anabasine, nicotine, and nornicotine and one species, *N. glauca*, had anabasine only. Smith & Smith (1942) give the alkaloidal composition of twenty-nine species, finding more with nicotine and nornicotine together than the Russian authors. Environmental and intraspecific genetic factors may cause large variations in the ratios of different alkaloids within a species, as in *Duboisia* spp. (Hills, Trautner & Rodwell, 1945*a*) and *Atropa belladonna* (Rowson, 1944*b*). *Nicotiana tabacum* has normally much nicotine with traces of nornicotine, but Markwood (1940) states that certain tobaccos selected for low nicotine content show a corresponding increase in nornicotine, up to 95 % of the total alkaloid. Anabasine is reported to be a very effective insecticide; its presence in *N. glauca* has therefore aroused much interest. Its present source is *Anabasis aphylla* (Chenopodiaceae), which grows in the arid regions around the Caspian Sea and appears not to be available in large quantities. Kostov (1943) reports that the range in alkaloid content in crosses within the genus *Nicotiana* is greater in the progeny than in the parents and anabasine formation dominant over nicotine formation in hybrids between *N. glauca* and the nicotine-forming species *N. tabacum* and *N. rustica*. This agrees with Ternovski, Khmura & Zhukov (1937), Zhukov (1939), Kuzmenko & Tikhvinskaya (1940) and Smith & Smith (1942), but Dawson (1945*a*, *b*) states that the anabasine of these authors is, in fact, a mixture of anabasine and nornicotine, mostly the latter. Kovalenko (1934) found both the protein and alkaloid contents of *N. tabacum* and *N. glauca* intermediate between those of the parents. Shmuk (1937) reports that the F_1 often only has one alkaloid, but in later generations plants appear carrying mixtures of the alkaloids found in the parents.

(*b*) *Studies on grafted plants.* The technique of grafting plants producing different alkaloids is not new, having been used by Strasburger (1885), though Mayer & Schmidt (1907) could not confirm his finding atropine in the tubers of potato acting as stock to a *Datura stramonium* scion. Its widespread use is, however, a recent development. Dawson (1942*a*) and Pal & Nath (1944) showed that tobacco scions on tomato stocks grow normally but produce no nicotine. In the reciprocal graft the tomato scion has much nicotine in the leaves and some in the stems and fruits. Excised tobacco roots continue to synthesize nicotine after repeated subculturing; it is abundant in tobacco leaves rooted in sand (Dawson, 1941, 1942*b*). Dawson considered that only the roots of tobacco synthesize nicotine—a conclusion reached, on similar evidence, by Mothes & Hieke (1943) and in agreement with the results of various other workers, though often not drawn by them. Grafe & Linsbauer (1906) showed that *Nicotiana affinis* has characteristically a low nicotine content, but much is found in its leaves when grafted to *N. tabacum* either as stock or scion. Shmuk, Smirnov & Ilyin (1941) found nicotine in *Solanum nigrum*, *Datura stramonium* and tomato grafted on *Nicotiana rustica* or *N. tabacum* stocks and none in the tobacco grafted reciprocally. Shmuk, Kostov & Borozdina (1939) state that *N. tabacum* on *N. glauca* stock has anabasine only, but consider that the stock has induced a change in the alkaloid of the scion. The data, however, are consistent with nicotine being formed in roots only and anabasine in both leaves and roots, as reported by Dawson (1944*b*). Shmuk (1940) reports similar results for grafts of *N. tabacum* and *N. rustica* with *N. glauca*. Kuzmenko & Tikhvinskaya (1940) and Kostov (1943) found downward movement of anabasine from *N. glauca* scions. Interpretation of these data is complicated by Dawson's (1945*a*, *b*) statement that the anabasine of the Russian authors is actually mostly nornicotine.

Daniel & Potel (1925) report grafting experiments with bittersweet and belladonna. The belladonna root grafted to a bittersweet scion had about half the solids content of a control and much reduced cellulose and atropine. Solanine passed from the bittersweet scion into the belladonna stock and atropine in the opposite direction. The grafted bittersweet had higher solids than a control, with a considerable increase in cellulose and in lignification. The general metabolic disturbance in this graft was greater than that met with, or at least reported by, most of the workers in this field, but the data suggest that atropine is formed in the belladonna root. Cromwell (1943*a*), in grafting experiments with belladonna and tomato, found free upward and very restricted downward movement of alkaloid. Krayevoy & Nechaev (1941) showed by physiological tests on a human subject that fruits of defoliated tomato scions on *Datura stramonium* stocks contain atropine. Kerkis & Pigulevskaya (1941) confirmed this work and showed that the atropine content of the tomato scion was lowest in the fruit, which was normal morphologically. Excised *D. stramonium* roots form hyoscyamine in culture; it passes to tomatoes grafted to *Datura* stocks (Dawson, 1944*a*; Peacock, Layerle & Dawson, 1944). Dawson (1945*a*, *b*) also studied nornicotine, which in *Nicotiana glutinosa* and *N. glauca* is formed in the leaves only, arising, probably by transmethylation, from nicotine translocated from the roots. Isolated roots of *Lupinus angustifolius* synthesize lupanine, using nitrate as their nitrogen source (Mothes & Kretschmer, 1946). Shmuk (1945) quotes work, not published elsewhere, by Moshkov showing that peas and sweet (non-alkaloidal) lupins on normal lupin stocks contain lupin alkaloids. He stresses the great mutual influence of stock and scion, illustrated by the formation (Shmuk, Pisarev & Vinogradova, 1944) in grain from wheat plants grown from embryos transplanted to rye endosperms of appreciable amounts of a trifructosan found in rye and not normally in wheat. Leaves from a belladonna scion on tomato stock not only contain no hyoscyamine but fail to form it in culture conditions inducing synthesis in normal detached belladonna leaves (Anon. 1945). This suggests that leaves of grafted scions lack some of the synthetic abilities of normal leaves as well as receiving different supplies of raw materials from the roots.

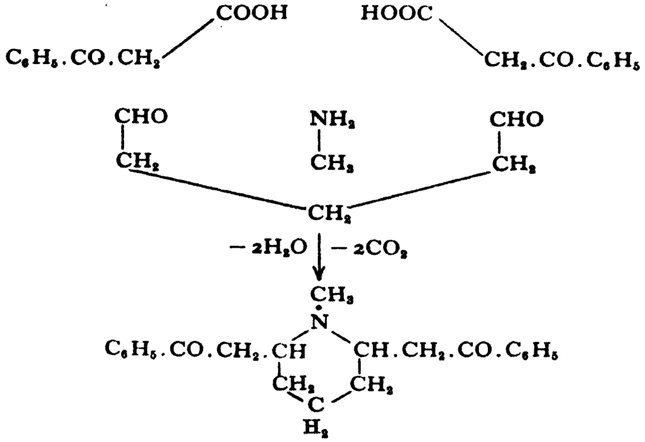
(*c*) *Evidence from* in vitro *studies*. Two general lines have been followed in studying routes of alkaloid synthesis in the plant. The schools of Robinson (1936) and of Schöpf (1937) have synthesized *in vitro* a number of alkaloids in 'physiological' or 'zellmöglich' conditions, i.e. at temperatures and hydrogen-ion concentrations within the range found in the plant cell. A number of other workers have supplied alkaloid-producing plants with substances suspected of being used in alkaloid synthesis, increased alkaloid content being taken as evidence of such use. Both methods yield interesting results. The second gives more direct information about synthesis in the plant, but even its results are suggestive rather than certain, as a substance might conceivably increase alkaloid content by a sparing action on the true precursor rather than directly.

Schöpf (1937) points out that Robinson's synthesis of tropinone dicarboxylic acid from succinic dialdehyde, methylamine and acetonedicarboxylic acid can be varied by use of mesotartaric dialdehyde, which gives in 'physiological conditions' a ketone teloidinone (Fig. 6) which, if its CO group is replaced by CHOH, gives the teloidine found esterified with tiglic acid in *Datura meteloides*. Similarly, lobelanine of *Lobelia inflata* can be synthesized in 'physiological conditions' from glutaric dialdehyde, methylamine and benzoylacetic acid (Fig. 7). β-(3, 4-dihydroxyphenyl)-ethylamine with acetaldehyde at pH 5 gives 1-methyl-6, 7-dihydroxy-1, 2, 3, 4-tetrahydroisoquinoline, closely related to the naturally occurring salsoline and carnegine (Fig. 8). Tryptamine with acetaldehyde at pH 5–6 and 25° C. gives tetrahydroharman (Fig. 9). Harman, found in *Symplocos racemosa* (Symplocaceae), is also related to the alkaloids of *Peganum harmala* (Zygophyllaceae) (Henry, 1939). Dihydroharman condenses with *o*-aminobenzaldehyde to



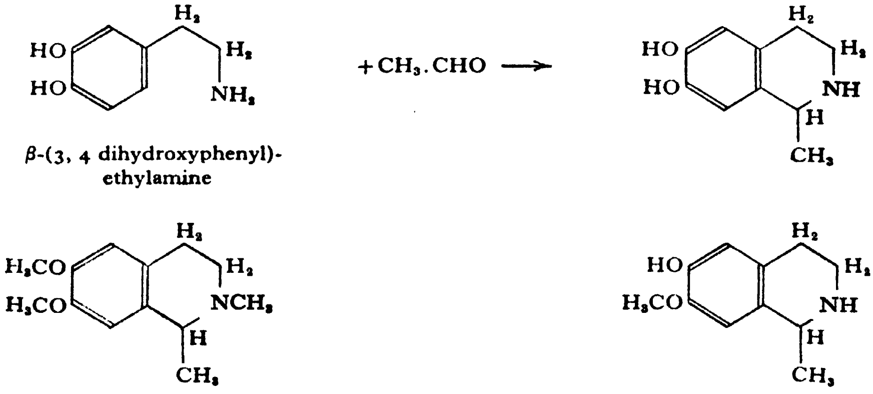
Teloidinone

Fig. 6



Lobelanine

Fig. 7

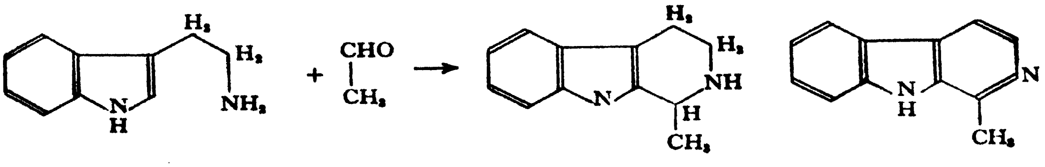


β-(3, 4 dihydroxyphenyl)-ethylamine

Carnegine

Salsoline

Fig. 8

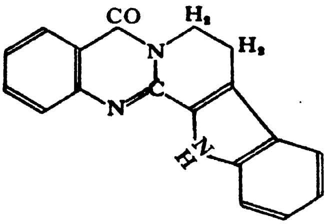


Tryptamine

Harman

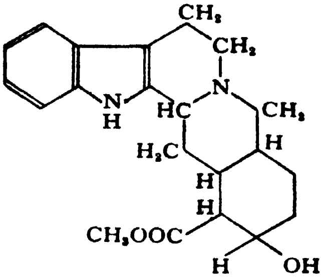
Fig. 9

a product which can be oxidized by potassium ferricyanide at 25° C. and pH 7 to rutaecarpine, found in *Evodia rutaecarpa* (Rutaceae) (Fig. 10). Hahn & Werner (1935) and Hahn, Bärwald, Schales & Werner (1935) also synthesized tetrahydroharman derivatives in very mild conditions, and obtained a compound with the complex yohimbine skeleton (Fig. 11) from tryptamine and *m*-hydroxyphenylpyruvic acid.



Rutaecarpine

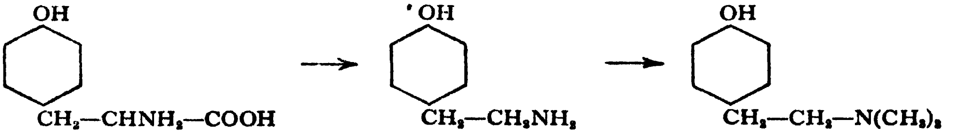
Fig. 10



Yohimbine

Fig. 11

Many of the intermediates used in these syntheses may plausibly be supposed to be formed in the cell from amino-acids. The production of amines from amino-acids by bacteria is well known, and methylated amines such as hordenine, known from several grasses, and *Anhalonium fissuratum* (Cactaceae) (Raoul, 1937*a*) and tetramethylputrescine (Wilstätter & Heubner, 1907) occur in higher plants. Raoul (1937*a*, *b*) showed that hordenine is synthesized rapidly in barley seedlings and produced some chemical evidence that it arises from tyrosine via tyramine (Fig. 12). Aldehydes could arise by deamination of amino-acids, e.g. succinic and glutaric dialdehydes, by a double deamination of ornithine and lysine respectively. Schöpf (1937) quotes earlier work by Kotake showing that tryptophane breakdown in plants and animals follows the sequence:



Tyrosine Tyramine Hordenine

Fig. 12

tryptophane → kynurenine → *o*-aminobenzoylpyruvic acid → kynurenic acid,

and suggests *o*-aminobenzoylacetic acid and *o*-aminobenzaldehyde may be side products from intermediates between these stages. Hypophorine, known from several *Erythrina* species (Leguminosae), is closely related to tryptophane (Henry, 1939). Anthranilic acid, another possible alkaloid intermediate, is a known plant constituent (Karrer, 1938).

Frey-Wyssling (1938) points out that in fusel-oil fermentation leucine is converted to isoamyl alcohol by loss of carbon dioxide and ammonia and addition of water; if the last step is omitted an olefine would be formed. He regards such olefines as intermediates in the formation from amino-acids of many secondary plant products (see p. 35), and suggests that aminobutylene from ornithine could give rise to the tobacco alkaloids, and aminoamylene from lysine to the pyridine and piperidine alkaloids. Barger, Martin & Mitchell (1938) found nortropine in *Duboisia myoporoides* esterified by isovaleric and α-methylbutyric acids and considered 'It is probable that the two acids are derived respectively from leucine and isoleucine, via the corresponding amyl alcohols, and we thus have some indication, not otherwise available, that these aliphatic amino acids may be used in the biogenesis of alkaloids.'

Solanine, found in potato and some other Solanaceae, is a glucoside of an alkaloid solanidine, which, like the *Veratrum* alkaloids from the Liliaceous species *Veratrum album*, *V. viride* and *Schoenocaulon officinale*, has the same carbon skeleton as the sterols (Soltys & Wallenfels, 1936; Craig & Jacobs, 1943*a*, *b*). They may be more closely related metabolically to the sterols than to the amino-acids. *Solanum commersonii*, suggested for potato-breeding work, has about seventy times as much solanine in its tubers as *S. tuberosum*, and is therefore definitely toxic (Wolf & Duggar, 1946). Henry (1939) stresses that *in vitro* syntheses of alkaloids in 'physiological conditions' commonly involve acetone or acetonedicarboxylic acid. The former is found in plants, but when present appears to be disposed of as the acetonecyanhydrin glucoside, phaseolunatin. The more reactive acid derivative appears not to have been found in plants.

Feeding experiments with possible alkaloid intermediates

Cromwell (1943*b*) found in *Atropa belladonna* an enzyme oxidizing putrescine (1, 4-diaminobutane) to an aldehyde and ammonia and suggests that the synthesis of the tropane alkaloids takes place as in Fig. 13. He found putrescine in *Atropa belladonna* and *Datura stramonium*; tetramethylputrescine occurs in *Hyoscyamus muticus* (Willstätter & Heubner, 1907). Injection of putrescine, arginine or hexamethylenetetramine increased the alkaloid content, the increase being fairly large with putrescine in *Atropa* and small elsewhere (Cromwell, 1943*a*). In most of the successful trials glucose also was injected. Cromwell's scheme for the synthesis of the tropane alkaloids is criticized in detail by

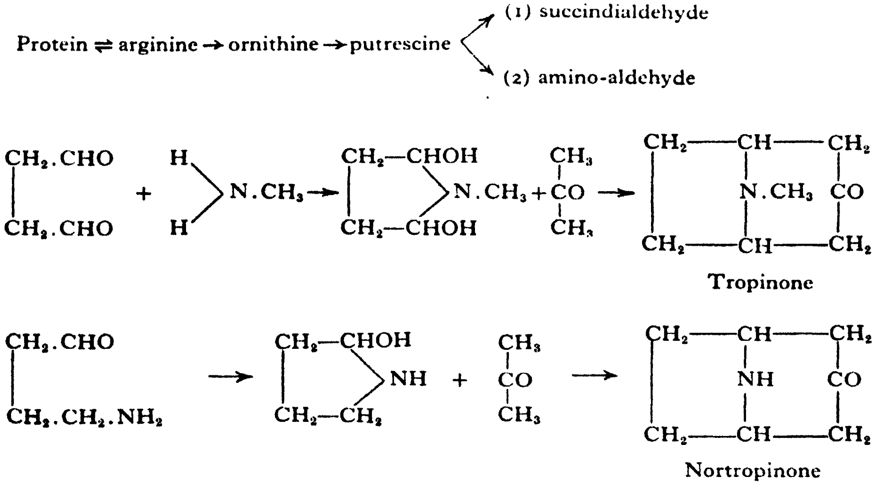
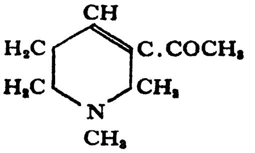


Fig. 13. (By reduction and esterification with tropic (α-hydroxymethyl-phenylacetic) acid tropinone and nortropinone would give respectively hyoscyamine and norhyoscyamine.)

Trautner (1947). James (1946*b*) concluded from feeding experiments with *Atropa belladonna* that the nitrogen of the tropane alkaloids comes from the δ-amino group of the arginine-ornithine group of amino-acids, and that the ring nitrogen of proline and α-amino-nitrogen are not available. Ugrymov (1940) suggests, on purely chemical grounds, arginine as the precursor of arecoline (Fig. 14), found in the palm *Areca catechu*.



Arecoline
Fig. 14

Dawson (1939) confirmed the finding of Klein & Linser (see McKee, 1937) that proline increases the nicotine content of excised tobacco leaves. Nicotinic acid, pyrrolidonecarboxylic acid and glutamic acid gave smaller increases, that with nicotinic acid being due entirely to its influence on growth and dry-matter accumulation. Glycine, arginine and α-amino-*n*-valeric acid were ineffective. Ciferri & Pratesi (1946) found that in tobacco seedlings grown without a supply of nitrogen, nicotinic acid, nicotinamide and methyl nicotinate increased dry weight but reduced nicotine content almost by half. They attributed this to diversion of nitrogen from alkaloid to protein formation, but made no protein determinations in confirmation. When nitrogen was fully supplied Pratesi, Ciferri & Cambieri (1946) found that all three compounds increased the nicotine and total alkaloid content about three times compared with controls, protein nitrogen being about the same. Ciferri (1946) found that pyridine tartrate increased the alkaloid content of *Nicotiana tabacum* and *N. rustica*. These data suggest that the pyridine nucleus and nicotinic acid can in tobacco be converted to nicotine. Nicotinic acid was first isolated from rice (Suzuki, Shimamura & Odake, 1912). Its distribution has been much studied, as it is a dietary essential for animals and a component of the cozymase molecule. It is essential for growth of excised pea roots (Addicot & Devirian, 1939) and probably occurs in all plants, though only a few form nicotine. Less is known about the occurrence of pyridine, but it is reported, together with the volatile bases *N*-methylpyrrolidine and *N*-methylpyrroline, in *Atropa belladonna* and *Datura stramonium* (Cromwell, 1943*a*). The specific mechanisms for nicotine formation thus seem concerned with the later stages of its synthesis rather than with the formation of possible precursors. De Tempe (1945) found alkaloid formation by the ergot fungus *Claviceps purpurea* erratic and affected by many factors, not all of which have been defined. Alkaloid formation was not increased by adding indole, skatole or tryptophane to the culture, though lysergic acid, a characteristic constituent of the ergot alkaloids, is an indole derivative.

Duboisia *alkaloids*

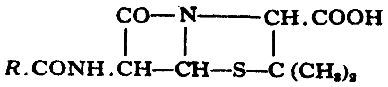
Australian species of the genus *Duboisia* (Solanaceae) have in recent years become a major commercial source of the tropane group of alkaloids, and some physiological and biochemical work has been done with them. They are trees, a habit unusual in the family. Hills *et al.* (1945*a*) showed that though there can be a very marked variation in the nature of the alkaloids of a single tree at different times, hyoscine predominates in *D. myopo-*

roides in the northern part of its range and hyoscyamine in the southern part. Hyoscyamine also predominates in *D. leichhardtii*, whose restricted range is in the northern part of that of *D. myoporoides*. Occasional trees of both species have appreciable amounts of atropine and norhyoscyamine. Young seedlings of both species contain hyoscine, the only alkaloid present in quantity up to 5 months, but at 9 months seedlings yield mixtures of hyoscine and hyoscyamine in the proportions expected of mature trees of their respective seed lines (Hills & Rodwell, 1946). The variability of the alkaloids in *D. myoporoides* was noted also by Petrie (1917), who suggested that it may produce both hyoscine and hyoscyamine, the balance between the two alkaloids being determined by growing conditions. Trautner (1947) postulates two separate alkaloid-forming systems, producing respectively hyoscine and hyoscyamine, each with its own set of related minor alkaloids, and with rather different esterification mechanisms. Some individual trees show a fairly stable alkaloid composition, others extreme fluctuations, e.g. a tree which had, in October, 3% of almost pure hyoscyamine had, in April, the same amount of almost pure hyoscine. No relation to environmental factors has been established, except for the geographical relation between locality and the dominant alkaloid in *D. myoporoides*. Tomato scions grafted to hyoscine-producing *D. myoporoides* stock contain large amounts of hyoscine but flower and fruit normally (Hills, Trautner & Rodwell, 1945*b*). *Nicotiana tabacum* on the same stock had a little hyoscine and much free tropine (a component of hyoscyamine). This suggests that the basic component is formed in the roots but esterified in the leaves. The esterifying mechanisms are very specific, e.g. in *Duboisia* and other Solanaceae scopine is always esterified with tropic acid as hyoscine, hydroxytropine with isovaleric acid and ψ-tropine with tiglic acid (*cis*-1 : 2-dimethylacrylic acid) (Trautner, 1947), nortropine with α-methylbutyric acid or isovaleric (β-methylbutyric) acid (Barger *et al.* 1938). In *Convolvulus pseudocantabricus*, however, tropine and nortropine are esterified with veratric (3, 4-dimethoxybenzoic) acid (Orekhov & Konovalova, 1934, 1935) and in *Erythroxylon coca* ψ-tropine with benzoic acid as tropacocaine (Karrer, 1938). Trautner (1947) points out that tiglic, isovaleric and α-methylbutyric acids are all structurally related to isoprene, the probable building unit for the terpenes. Tropic acid is also related, at least formally, to the terpenes; all acids esterifying tropane bases in solanaceous plants may thus be connected with terpene metabolism. The triterpenoid ursolic acid occurs in comparatively large amounts in leaves of all three *Duboisia* species (Trautner & Neufeld, 1947). The esterifying acids of the *Veratrum* (Liliaceae) alkaloids also include tiglic, α-methylbutyric and veratric, together with methylethylglycollic and acetic (Krayer & Acheson, 1946). Some *Erythrina* alkaloids are esterified with sulphoacetic acid (Folkers, Konuiszy & Shavel, 1944). Many similar instances of specific esterification of alkaloids could be cited. The complexity and specificity of the esterification effects raise difficult biochemical problems.

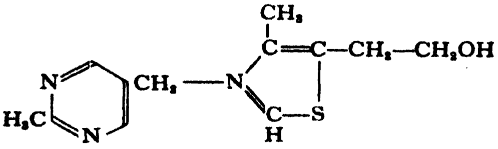
A third *Duboisia*, *D. hopwoodii*, has alkaloids resembling those of the *Nicotianas*, but shows the variability in alkaloid composition which seems typical of the genus. Some samples have nicotine as the main alkaloid, with little or no nornicotine (Bottomley, Nottle & White, 1945; Trautner & Neufeld, 1946), and others nornicotine with little or no nicotine (Hicks & Le Messurier, 1935; Späth, Hicks & Zajic, 1935; Hicks and Sinclair, 1947).

Several observations on the effect on alkaloid formation of environmental conditions and nutrient supply are scattered through the literature. Grebinski (1941) found increased carbohydrate, protein and alkaloid contents in *Nicotiana rustica* grown at high altitudes. Alkaloid increased relatively more than protein. Shading decreases nicotine content in *N. tabacum*; much of the effect is attributable to higher water content in the leaves (Horowitz, 1933). Tubers of potatoes grown in hot districts have up to four times as much solanine as those grown from the same batch of seed in cool mountainous areas (Arutyunyan, 1940).

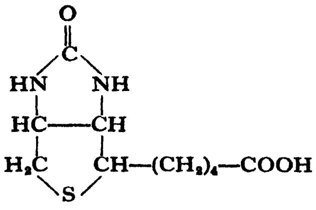
High nitrogen supply tends to increase nicotine content in tobacco, and high potassium supply to decrease it (Garner *et al.* 1934; Horowitz, 1942). Similar results are reported for atropine in belladonna (Anon. 1945), nitrate being somewhat more effective than ammonium. Vladimirov (1939), however, found the nicotine content of *N. rustica* much greater with ammonium than with nitrate supply. Both nitrogen and potassium are stated (Prasad, 1946) to favour alkaloid synthesis in *Hyoscyamus niger*. The alkaloid content of belladonna rises with increased phosphorus supply (Anon. 1945).



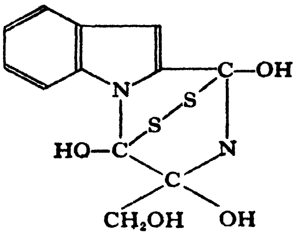
Penicillin, general formula
R = *p*-hydyoxybenzyl in one individual penicillin.



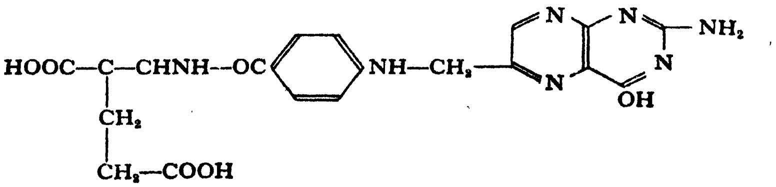
Thiamine



Biotin



Gliotoxin, from *Trichoderma viride* and other moulds



Pteroylglutamic acid

Fig. 15

In *Datura stramonium*, *D. tatula* (Rowson, 1944*a*), *Nicotiana rustica* and *N. tabacum* (Noguti, Okuma & Oka, 1939; Noguti, Oka & Ôtuka, 1940), tetraploid plants have a higher alkaloid content than diploids.

These results allow few generalizations on the effect of environmental conditions on alkaloid content. Some effects seem to vary from one species to another. Those induced by variations in mineral supply are in general small, though in some cases shown to be statistically significant.

Structural formulae of several other nitrogenous plant constituents of general interest are shown in Fig. 15. They suggest that, while the ingenuity of the organic chemist has produced several classes of nitrogen compounds unknown to organisms, the synthetic activities of plant cells can still hold surprises for him. Little work has been reported on the mechanism of synthesis of these complex molecules; penicillin production is increased by supply of phenylacetic acid (Anon. 1947).

METABOLIC RELATIONS OF UREA

(*a*) *Animals*. The Krebs-Henseleit ornithine cycle (for earlier references see McKee (1937)) retains its place as the most generally accepted formulation of the processes leading to urea formation in the animal. Bicarbonate labelled with C^{13} (Rittenberg & Waelsch, 1940) and C^{11} (Evans & Slotin, 1940) contributes approximately its predicted share of the carbon of urea formed from ornithine by liver slices. Bloch (1946), using N^{15}, has shown rapid regeneration in liver slices of the amidine group of arginine, in agreement with the ornithine cycle. Gornall & Hunter (1943) also report evidence in its favour.

Glutamic acid is essential for urea formation, being apparently involved in the introduction of ammonia at the citrulline-arginine stage of the cycle (Cohen & Hayano, 1946*a*, *b*, 1947). Borsook and Dubnoff (1941) also noted that aspartic and glutamic acids, and compounds readily converted to them, stimulate urea formation in kidney slices. Leuthardt & Glasson (1942*b*) found marked stimulation of urea formation in liver slices by pyruvic acid, oxalacetic acid, glutamine and asparagine. They suggest a scheme for citrulline synthesis via the unknown half-amide of oxalacetic acid; an alternative explanation of their observations would be increased glutamic acid formation from the compounds added, via the tricarboxylic acid cycle and associated reactions. Ratner (1947) reports in a preliminary note reactions, stated to be enzymatically catalysed, whereby citrulline condenses with aspartic acid to a product which later breaks down to arginine and malic acid. The reaction, like that involving glutamic acid (Cohen & Hayano, 1946*b*), requires high-energy phosphate.

Ornithine seems unknown as a protein constituent, but is reported (Gordon, Martin & Synge, 1943; Brian, 1947) among the hydrolysis products of tryocidine and gramicidin-S, bacterial antibiotic polypeptides.

(*b*) *Plants*. Evidence for the operation of the ornithine cycle is much less complete in plants than in animals. Srb & Horowitz (1944) describe seven genetically and biochemically distinct arginine-requiring mutant strains of the fungus *Neurospora crassa*. They fall into three classes: using arginine, ornithine or citrulline; using arginine or citrulline but not ornithine; using arginine only. In this species, therefore, there is a strong presumption that the ornithine cycle follows the same general course as in mammalian liver. Granick (1938*b*) concludes from a study of its distribution in the soy bean that urease is connected with protein synthesis.

Thiourea occurs in traces in healthy and in much larger amounts in rust-infected leaves of *Rubus saxatilis*, *Alchemilla vulgaris* and *Rhamnus cathartica*. Leaves kept with their petioles in dilute thiourea solutions show much accelerated chlorophyll breakdown compared with controls in distilled water. *Botrytis cinerea* forms thiourea when supplied with ammonium or asparagine, but not with nitrate (Ovcharov, 1937). *Verticillium dahliae* and *Pythium* sp. also form thiourea in culture (Zelenin, 1939). Thiourea, and in very much lower concentrations phenyl and α-naphthyl thioureas, inhibit potato tyrosinase (Dubois & Erway, 1946).

PURINE METABOLISM

Fosse and his school in France have continued their studies (see McKee, 1937) on the purine metabolism of plants. The chief metabolites are uric acid, allantoin (known as a plant constituent since the early work of Richardson & Crampton (1886)), and allantoic acid. Brunel & Echevin (1937) showed that purine nitrogen increases in germination in *Agrostemma githago*, as found in wheat by Oparin & Gelman (1946). The course of purine breakdown seems to vary between species. Soy beans (Echevin & Brunel, 1937*b*) contain the enzymes allantoinase, forming allantoic acid from allantoin, and allantoicase, forming glyoxylic acid and urea from allantoic acid. *Lupinus albus* lacks allantoicase, and allantoic acid accumulates during germination (Echevin & Brunel, 1937*a*). Allantoicase has been demonstrated also in fungi and animal tissues (Brunel, 1937). In wheat and maize seedlings allantoinase is weak and allantoin accumulates during germination (de Graeve, 1937). The radish lacks allantoin, allantoic acid and the corresponding enzymes but can use allantoin as a nitrogen source (Brunel & Echevin, 1939). Supply of allantoin to soy beans in sterile culture gives large increases in amino-acids and amides and a smaller increase in protein (Echevin, & Brunel 1939). The evidence cited refers mostly to seedlings, but allantoic acid occurs also in mature leaves of the hazel (*Corylus avellana*) (Leroux, 1937). Fosse (1939) reaffirms the conclusion that in higher plants (but not in fungi) urea is an end-product of purine catabolism. Little is known of purine synthesis in either animals or plants. Both the pigeon and the rat fail to use ingested purines and pyrimidines in nucleoprotein synthesis, metabolizing them to urea and ammonia (Plentl & Schoenheimer, 1944).

CYANIDE METABOLISM

Although cyanogenetic glucosides are known to occur in many plants scattered through different families, the metabolic relationships of cyanide are obscure. Frey-Wyssling (1942) states that cyanogenetic glucosides are localized in the vacuole and suggests they are water-soluble storage derivatives of insoluble substances. Léeman (1935) lists eighty-eight grasses reported to contain hydrocyanic acid, and quotes several early workers who state the cyanide content of plants increases with the nitrogen supply of the soil, as confirmed by Pinckney (1924), Thorp, Deem, Harrington & Tobiska (1937) and Boyd, Aamodt, Bohstedt & Truog (1938). Young plants, and especially new shoots from established plants, have high cyanide contents (Boyd *et al.* 1938; Winks, 1940), suggesting an association with active metabolism. Sanford (1914) reports filling holes in the trunks of broom and peach trees with solid potassium cyanide. Scale insects infesting them thereupon died and the trees became very vigorous. Fruits of citrus and olive trees fumigated with gaseous hydrocyanic acid occasionally develop characteristic ridges due to irregular and excessive local growth of the skin (Sinclair & Lindgren, 1943, 1946). In *Trifolium repens* presence or absence of cyanoglucoside is due to a simple pair of genetic factors (Williams, 1939). Other properties of the plant seem largely unaffected by the presence of cyanide.

CONCLUSION

Improvement in knowledge of protein catabolism has been essentially a consolidation of positions already held in 1937. The synthesis of protein, and especially its regulation, still present obscure features, but some progress has been made towards their elucidation. The mechanisms of amino-acid and peptide synthesis, and the interactions between protein, carbohydrate and fat metabolism, are beginning to be understood. The basic importance of mechanisms of energy transfer from exergonic oxidations to endergonic syntheses is now realized, and some evidence suggests that phosphorylation systems are involved in protein synthesis. Notable advances have also been made in the study of symbiotic nitrogen fixation and of the assimilation of nitrate.

The MS. of this review has been read and criticized by Dr J. R. Vickery, Chief of the Division of Food Preservation and Transport, Council for Scientific and Industrial Research, Dr R. N. Robertson, Officer-in-Charge of the Fruit Storage Section of that Division, and Prof. J. G. Wood, Botany Department, University of Adelaide. I wish to thank them for their co-operation; also Miss B. Johnston, Divisional Librarian, whose knowledge of the resources of Australian libraries has greatly facilitated preparation of the bibliography.

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REFERENCES

Ackermann, D. & Appel, W. (1940). Über stickstoffhaltige Bestandteile der Jackbohne. *Hoppe-Seyl. Z.* **262**, 103.

Addicott, F. T. & Devirian, P. S. (1939). A second growth factor for excised pea roots: nicotinic acid. *Amer. J. Bot.* **26**, 667.

Addoms, R. M., Nightingale, G. T. & Blake, M. A. (1930). Development and ripening of peaches as correlated with physical characteristics, chemical composition, and histological structure of the fruit flesh. II. Histology and microchemistry. *Bull. N.J. agric. Exp. Sta.* no. 507.

Adler, E., Das, N. B., von Euler, H. & Heyman, U. (1938). Biologische Dehydrierung und Synthese der Glutaminsäure. *C.R. Lab. Carlsberg*, Ser. Chim., **22**, 15.

Adler, E., Günther, G. & Everett, J. E. (1938). Über den enzymatischen Abbau und Aufbau der Glutaminsäure. IV. In Hefe. *Hoppe-Seyl. Z.* **255**, 27.

Adler, E., Hellström, V., Günther, G. & von Euler, H. (1938). Über den enzymatischen Abbau und Aufbau der Glutaminsäure. III. In *Bacterium coli. Hoppe-Seyl. Z.* **255**, 14.

Adler, E., von Euler, H., Günther, G. & Plass, M. (1939). Isocitric dehydrogenase and glutamic acid synthesis in animal tissues. *Biochem. J.* **33**, 1028.

Ahlgren, H. L. & Aamodt, O. S. (1939). Harmful root interactions as a possible explanation for effects noted between various species of grasses and legumes. *J. Amer. Soc. Agron.* **31**, 982.

Albaum, H. G. & Cohen, P. P. (1943). Transamination and protein synthesis in germinating oat seedlings. *J. biol. Chem.* **149**, 19.

Alberts-Dietert, F. (1941). Die Wirkung von Eisen und Mangan auf die Stickstoffassimilation von *Chlorella. Planta*, **32**, 88.

Alcock, R. S. (1936). The synthesis of proteins *in vivo. Physiol. Rev.* **16**, 1.

Ali-Zade, M. (1941). Assimilation des Stickstoffs der Knöllchen der Leguminosen. *C.R. Acad. Sci. U.R.S.S.* **30**, 256.

Allison, F. E., Hoover, S. R. & Minor, F. W. (1942). Biochemical nitrogen fixation studies. IV. Experiments with excised legume nodules. *Bot. Gaz.* **104**, 63.

Allison, F. E., Hoover, S. R. & Morris, H. J. (1937). Physiological studies with the nitrogen-fixing alga, *Nostoc muscorum. Bot. Gaz.* **98**, 433.

Allison, F. E., Ludwig, C. A., Hoover, S. R. & Minor, F. W. (1939). Legume nodule metabolism and nitrogen fixation. *Nature, Lond.*, **144**, 711.

Allison, F. E., Ludwig, C. A., Hoover, S. R. & Minor, F. W. (1940). Biochemical nitrogen fixation studies. I. Evidence for limited oxygen supply within the nodules. *Bot. Gaz.* **101**, 513.

Alten, F., Goeze, G. & Fischer, H. (1937). Kohlensäureassimilation und Stickstoffhaushalt bei gestaffelter Kaligabe. *Bodenk. Pflanz.* **5** (50), 259.

Ames, S. P., Sarma, P. S. & Elvehjem, C. A. (1947). Transaminase and pyridoxine deficiency. *J. biol. Chem.* **167**, 135.

Amos, G. L. & Wood, J. G. (1939). The effects of variation in nitrogen-supply and water content on the amounts of carbohydrates in the leaves of grass plants. *Aust. J. exp. Biol. med. Sci.* **17**, 287.

Anderson, A. J. (1946). Molybdenum in relation to pasture improvement in South Australia. *J. Coun. sci. industr. Res. Aust.* **19**, 1.

Anderson, A. J. & Oertel, A. C. (1946). Plant responses to molybdenum as a fertilizer. 2. Factors affecting the response of plants to molybdenum. *Bull. Coun. sci. industr. Res. Aust.* no. 198.

Anderson, A. J. & Thomas, M. P. (1946). Plant responses to molybdenum as a fertilizer. 1. Molybdenum and symbiotic nitrogen fixation. *Bull. Coun. sci. industr. Res. Aust.* no. 198.

Anfinsen, C. B., Beloff, A., Hastings, A. B. & Solomon, A. K. (1947). The *in vitro* turnover of dicarboxylic amino acids in liver slice proteins. *J. biol. Chem.* **168**, 771.

Anonymous (1945). *Ann. Rep. Oxford Medicinal Plants Scheme.*

Anonymous (1947). The chemical study of penicillin. A brief history. *Science*, **105**, 653.

Archbold, H. K. (1945). Some factors concerned in the process of starch storage in the barley grain. *Nature, Lond.*, **156**, 70.

Archibald, R. M. (1945*a*). Note on the reported formation of urea from glutamine by liver extracts, and on the preparation of glutamine free from arginine. *J. biol. Chem.* **159**, 693.

Archibald, R. M. (1945*b*). Chemical characteristics and physiological roles of glutamine. *Chem. Rev.* **37**, 161.

Arenz, B. (1941). Beitrag zur physiologischen Auswirkung von Ammoniak und Nitratstickstoff. *Biochem. Z.* **308**, 196.

Arenz, B. (1942*a*). Über die Aufnahme und Verarbeitung von NO_3-N und NH_3-N durch Gramineenkeimlinge. *Bodenk. Pflanz.* **27**, 330.

Arenz, B. (1942*b*). Über die Aufnahme und Verarbeitung von NO_3-N und NH_3-N durch Leguminosenkeimlinge. *Bodenk. Pflanz.* **27**, 352.

Arnon, D. I. (1937). Ammonium and nitrate nitrogen of barley at different seasons in relation to hydrogen-ion concentration, manganese, copper and oxygen supply. *Soil Sci.* **44**, 91.

Arnon, D. I. (1938). Microelements in culture solution experiments with higher plants. *Amer. J. Bot.* **25**, 322.

Arnon, D. I. (1939). Effects of ammonium and nitrate nitrogen on the mineral composition and sap characteristics of barley. *Soil Sci.* **48**, 295.

Arnon, D. I. & Stout, P. R. (1939). Molybdenum as an essential element for higher plants. *Plant. Physiol.* **14**, 599.

Arutyunyan, L. A. (1940). Solanine content of potatoes. *Voprosy Pitaniya* (*Problems of Nutrition*), **5**, 30 (Russian).

Aso, K., Migita, M. & Ihda, T. (1939). The mechanism of nitrogen utilisation by *Azotobacter*. *Soil Sci.* **48**, 1.

Astbury, W. T. (1943). X-rays and the stoichiometry of the proteins. *Adv. Enzymol.* **3**, 63.

Aubel, E. (1938). Sur la réduction des nitrites par le bacille *coli*. *C.R. Soc. Biol., Paris*, **128**, 45.

Audus, L. J. & Quastel, J. H. (1947). Toxic effects of amino-acids and amines on seedling growth. *Nature, Lond.*, **160**, 222.

Avery, G. S. & Pottorf, L. (1945*a*). Auxin and nitrogen relationships in green plants. *Amer. J. Bot.* **32**, 666.

Avery, G. S. & Pottorf, L. (1945*b*). Polyploidy, auxin and nitrogen in green plant tissue. *Amer. J. Bot.* **32**, 669.

Bach, A. N., Yermoleva, Z. V. & Stepanian, M. P. (1934). Fixation of atmospheric nitrogen by enzymes extracted from *Azotobacter chroöcoccum*. *C.R. Acad. Sci. U.R.S.S.* **1**, 22 (Russian).

Baddiley, J. & Gale, E. F. (1945). Codecarboxylase function of 'pyridoxal phosphate'. *Nature, Lond.*, **155**, 727.

Bailey, K., Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). Critique of the Foreman method for the estimation of the dicarboxylic acids in protein hydrolysates. *Biochem. J.* **37**, 360.

Balch, R. T. (1945). Report on aconitic acid studies at Houma, La., Station. *Sugar Bull.* **23**, 197.

Bald, J. G. (1942). The effect of potato virus *X* on growth and yield. *Aust. J. Sci.* **4**, 177.

Ballard, L. A. T. & Petrie, A. H. K. (1936). Physiological ontogeny in plants and its relation to nutrition. *Aust. J. exp. Biol. med. Sci.* **14**, 135.

Banga, I. & Szent-Györgyi, A. (1937). Über die Bedeutung der Fumarsäure für die tierische Gewebsatmung. IV. Mitteilung. *Hoppe-Seyl. Z.* **245**, 113.

Barger, G., Martin, W. F. & Mitchell, W. (1937). The minor alkaloids of *Duboisia myoporoides*. *J. chem. Soc.* p. 1820.

Barger, G., Martin, W. F. & Mitchell, W. (1938). The minor alkaloids of *Duboisia myoporoides*. Pt II. *J. chem. Soc.* p. 1685.

Barnard, C. & Finnemore, H. (1945). Drug plant investigations. I. Progress report. *J. Coun. sci. industr. Res. Aust.* **18**, 277.

Barrenscheen, H. K. & von Vályi-Nagi, T. (1942). Die Methylierung durch pflanzliche und tierische Gewebe. I. Mitteilung. Methionin als Methylierungsagens bei der Synthese des Kreatins und Betains durch etiolierte Weizenkeimlinge. *Hoppe-Seyl. Z.* **277**, 97.

Barrien, B. S. & Wood, J. G. (1939). Studies on the sulphur metabolism of plants. II. The effect of nitrogen supply on the amounts of protein sulphur, sulphate sulphur and on the value of the ratio of protein nitrogen to protein sulphur in leaves at different stages during the life cycle of the plant. *New Phytol.* **38**, 257.

Bartlett, G. R. & Barron, E. S. G. (1947). The effect of fluoroacetate on enzymes and on tissue metabolism. Its use for the study of the oxidative pathway of pyruvate metabolism. *J. biol. Chem.* **170**, 67.

Beadle, G. W. (1945). Genetics and metabolism in *Neurospora*. *Physiol. Rev.* **25**, 643.

Beaumont, A. B., Larsinos, G. J., Pieкenbrock, P. & Nelson, P. R. (1931). The assimilation of nitrogen by tobacco. *J. agric. Res.* **43**, 559.

Bellamy, W. D., Umbreit, W. W. & Gunsalus, I. C. (1945). The function of pyridoxine: Conversion of members of the vitamin B_6 group into codecarboxylase. *J. biol. Chem.* **160**, 461.

Bennett, J. P. (1945). Iron in leaves. *Soil Sci.* **60**, 91.

Bergmann, M. & Fraenkel-Conrat, H. (1938). The enzymatic synthesis of peptide bonds. *J. biol. Chem.* **124**, 1.

Bergmann, M. & Fruton, J. S. (1938). Some synthetic and hydrolytic experiments with chymotrypsin. *J. biol. Chem.* **124**, 321.

Bergmann, M. & Fruton, J. S. (1944). The significance of coupled reactions for the enzymatic hydrolysis and synthesis of proteins. *Ann. N.Y. Acad. Sci.* **45**, 409.

Bertrand, D. (1939). Sur la diffusion du molybdène chez les végétaux. *C.R. Acad. Sci., Paris*, **208**, 2024.

Binkley, F. & du Vigneaud, V. (1942). The formation of cysteine from homocysteine by liver tissue of rats. *J. biol. Chem.* **144**, 507.

Blackman, G. E. (1938). The interaction of light intensity and nitrogen supply in the growth and metabolism of grasses and clover (*Trifolium repens*). I. The effects of light intensity and nitrogen supply on the clover content of a sward. *Ann. Bot., Lond.*, N.S. **2**, 257.

Blackman, G. E. & Templeman, W. G. (1938). The interaction of light intensity and nitrogen supply in the growth and metabolism of grasses and clover (*Trifolium repens*). II. The influence of light intensity and nitrogen supply on the leaf production of frequently defoliated plants. *Ann. Bot., Lond.*, N.S. **2**, 765.

Blackman, G. E. & Templeman, W. G. (1940). The interaction of light intensity and nitrogen supply in the growth and metabolism of grasses and clover (*Trifolium repens*). IV. The relation of light intensity and nitrogen supply to the protein metabolism of the leaves of grasses. *Ann. Bot., Lond.*, N.S. **4**, 533.

Blagoveshchenski, A. V. (1940). Proteases. In *Enzymes*, A. N. Bach & V. A. Engelhardt, Moscow-Leningrad (Russian).

Blake, M. A., Davidson, O. W., Addoms, R. M. & Nightingale, G. T. (1931). Development and ripening of peaches as correlated with physical characteristics, chemical composition, and histological structure of the fruit flesh. I. Physical measurements of growth and flesh texture in relation to the market and edible qualities of the fruit. *Bull. N.J. agric. Exp. Sta.* no. 525.

Blanchard, M., Green, D. E., Nocito, V. & Ratner, S. (1945). Isolation of *l*-amino acid oxidase. *J. biol. Chem.* **161**, 583.

Bloch, K. (1946). The metabolism of *l*-(+)-arginine in the rat. *J. biol. Chem.* **165**, 469.

Bloch, K. (1947). The metabolism of acetic acid in animal tissues. *Physiol. Rev.* **27**, 574.

Bloch, K. & Borek, E. (1946). Biological acetylation of natural amino acids. *J. biol. Chem.* **164**, 485.

Bond, G. (1938). Excretion of nitrogenous substances from leguminous root nodules; observations on soya bean. *Ann. Bot., Lond.*, N.S. **2**, 61.

Bond, G. (1941). Symbiosis of leguminous plants and nodule bacteria. II. Further observations on the excretion of nitrogenous substances from nodules. *Ann. Bot., Lond.*, N.S. **5**, 647.

Bond, G. & Boyes, J. (1939). Excretion of nitrogenous substances from leguminous root nodules; observations on various leguminous plants. *Ann. Bot., Lond.*, N.S. **3**, 901.

Bonner, J. (1946). The role of organic matter, especially manure, in the nutrition of rice. *Bot. Gaz.* **108**, 267.

Borovik, S. A., Bergman, G. G. & Borovik-Romanova, T. F. (1943). Trace elements of kok-saghyz. *C.R. Acad. Sci. U.R.S.S.* **40**, 239.

Borsook, H. & Dubnoff, J. W. (1940). The formation of creatine from glycocyamine in the liver. *J. biol. Chem.* **132**, 559.

Borsook, H. & Dubnoff, J. W. (1941). The conversion of citrulline to arginine in kidney. *J. biol. Chem.* **141**, 717.

Borsook, H. & Dubnoff, J. W. (1943). The metabolism of proteins and amino acids. *Ann. Rev. Biochem.* **12**, 183.

Bortels, H. (1930). Molybdän als Katalysator bei der biologischen Stickstoffbindung. *Arch. Mikrobiol.* **1**, 333.

Bortels, H. (1936). Weitere Untersuchungen über die Bedeutung von Molybdän, Vanadium, Wolfram und anderen Erdaschenstoffen für stickstoffbindende und andere Mikroorganismen. *Zbl. Bakt.* 2te. Abteil. **95**, 193.

Bortels, H. (1940). Über die Bedeutung des Molybdäns für stickstoffbindende Nostocaceen. *Arch. Mikrobiol.* **11**, 155.

Boswell, J. G. (1945). Oxidation systems in the potato tuber. *Ann. Bot., Lond.*, N.S. **9**, 55.

Boswell, J. G. & Whiting, G. C. (1938). A study of the polyphenol oxidase system in potato tubers. *Ann. Bot., Lond.*, N.S. **2**, 847.

Bottomley, W. B. (1909). Some effects of nitrogen-fixing bacteria on the growth of non-leguminous plants. *Proc. roy. Soc.* B, **81**, 287.

Bottomley, W. B. (1912). The root nodules of *Myrica gale*. *Ann. Bot., Lond.*, **26**, 111.

Bottomley, W., Nottle, R. A. & White, D. E. (1945). The alkaloids of *Duboisia Hopwoodii*. *Aust. J. Sci.* **8**, 18.

Bovarnick, M. (1942). The formation of extracellular *d*(−)-glutamic acid polypeptide by *Bacillus subtilis*. *J. biol. Chem.* **145**, 415.

Boyd, F. T., Aamodt, O. S., Bohstedt, G. & Truog, E. (1938). Sudan grass management for control of cyanide poisoning. *J. Amer. Soc. Agron.* **30**, 569.

Boynton, D. & Compton, O. C. (1944). The influence of differential fertilization with ammonium sulfate on the chemical composition of McIntosh apple leaves. *Proc. Amer. Soc. hort. Sci.* **45**, 9.

Bradley, W. B., Eppson, H. F. & Beath, O. A. (1940). Livestock poisoning by oat hay and other plants containing nitrate. *Bull. Wyoming agric. Exp. Sta.* no. 241.

Brand, E., Saidel, L. J., Goldwater, W. H., Kassel, B. & Ryan, F. J. (1945). The empirical formula of β-lactoglobulin. *J. Amer. chem. Soc.* **67**, 1524.

Braunstein, A. E. (1939). Die enzymatische Umaminierung der Aminosäuren und ihre physiologische Bedeutung. *Enzymol.* **7**, 25.

Braunstein, A. E. (1947). Transamination and the integrative functions of the dicarboxylic acids in nitrogen metabolism. *Adv. Protein Chem.* **3**, 1.

Braunstein, A. E. & Asarkh, R. M. (1945). The mode of deamination of *l*-amino acids in surviving tissues. *J. biol. Chem.* **157**, 421.

Braunstein, A. E. & Kritzmann, M. G. (1937*a*). Amino acid formation by intermolecular transfer of amino groups. I. The metabolism of *l*-(+)-glutamic acid in muscle tissue. *Biokhim.* **2**, 242 (Russian).

Braunstein, A. E. & Kritzmann, M. G. (1937*b*). Über den Ab- und Aufbau von Aminosäuren durch Umaminierung. *Enzymol.* **2**, 129.

Braunstein, A. E. & Kritzmann, M. G. (1946). Co-aminopherase, co-decarboxylase and pyridoxal. *Nature, Lond.*, **158**, 102.

Breakwell, E. J. & Hutton, E. M. (1939). A protein 'survey' of the South Australian wheat belt. *J. Aust. Inst. agric. Sci.* **5**, 103.

Brenchley, W. E. & Thornton, H. G. (1925). The relation between the development, structure and functioning of the nodules on *Vicia faba*, as influenced by the presence or absence of boron in the nutrient medium. *Proc. roy. Soc.* B, **98**, 373.

Breusch, F. L. (1943). Citric acid cycle; sugar and fat breakdown in tissue metabolism. *Science*, **97**, 490.

Brian, P. W. (1947). Penicillin and other antibiotics. *Nature, Lond.*, **160**, 554.

Briggs, G. B. (1939). Effect of boron in the substrate on the rate of nitrate absorption and on nitrogen distribution in nasturtium. *Plant Physiol.* **18**, 415.

Brown, R. (1946). Studies on germination and seedling growth. III. Early growth in relation to certain aspects of nitrogen metabolism in the seedling of barley. *Ann. Bot., Lond.*, N.S. **10**, 73.

Brunel, A. (1937). Un nouvel enzyme, l'allantoïcase. Sa présence dans la règne animal. *C.R. Acad. Sci., Paris*, **204**, 380.

Brunel, A. & Echevin, R. (1937). Évolution de l'azote, apparition de l'allantoïnase et de l'uréase dans les germinations de Nielle (*Agrostemma Githago* L.). *C.R. Acad. Sci., Paris*, **205**, 81.

Brunel, A. & Echevin, R. (1939). Sur l'assimilation de l'allantoïne par les plantes supérieures. *C.R. Acad. Sci., Paris*, **208**, 1043.

Buchanan, J. M., Sakami, W., Gurin, S. & Wilson, D. W. (1945). A study of the intermediates of acetoacetate oxidation with isotopic carbon. *J. biol. Chem.* **157**, 747.

Bucherer, H. & Enders, C. (1942). Abbau von Histamin und Nicotin durch Bacterien. *Biochem. Z.* **310**, 222.

Bull, H. B. (1941). Protein structure. *Adv. Enzymol.* **1**, 1.

Burk, D. & Horner, C. K. (1936). The origin and significance of ammonia formed by *Azotobacter*. *Soil Sci.* **41**, 81.

Burkhart, L. (1938). Ammonium nutrition and metabolism of etiolated seedlings. *Plant Physiol.* **13**, 265.

Burrill, T. J. & Hansen, R. (1917). Is symbiosis possible between legume bacteria and non-legume plants? *Bull. Ill. agric. Exp. Sta.* no. 202.

Burris, R. H. (1941). Failure of barley to fix molecular N^{15}. *Science*, **94**, 238.

Burris, R. H. (1942). Distribution of isotopic nitrogen in *Azotobacter vinelandii*. *J. biol. Chem.* **143**, 509.

Burris, R. H., Eppling, F. J., Wahlin, H. B. & Wilson, P. W. (1943). Detection of nitrogen fixation with isotopic nitrogen. *J. biol. Chem.* **148**, 349.

Burris, R. H. & Wilson, P. W. (1944). The metabolism of ammonia by *Azotobacter vinelandii*. *J. Bact.* **47**, 410 (abstract only).

Burris, R. H. & Wilson, P. W. (1945). Biological nitrogen fixation. *Ann. Rev. Biochem.* **14**, 685.

Burris, R. H. & Wilson, P. W. (1946*a*). Comparison of the metabolism of ammonia and molecular nitrogen in *Azotobacter*. *J. biol. Chem.* **165**, 595.

Burris, R. H. & Wilson, P. W. (1946*b*). Characteristics of the nitrogen-fixing enzyme system in *Nostoc muscorum*. *Bot. Gaz.* **108**, 254.

Burström, H. (1939*a*). Über die Schwermetallkatalyse der Nitratassimilation. *Planta*, **29**, 292.

Burström, H. (1939*b*). Die Rolle des Mangans bei der Nitratassimilation. *Planta*, **30**, 129.

Burström, H. (1943*a*). Photosynthesis and assimilation of nitrate by wheat leaves. *K. Lantbrukshögskolans Ann.* **11**, 1.

Burström, H. (1943*b*). Studies on the products of the photosynthesis. *Ark. Bot.* **30**B, 1.

Burström, H. (1945). The nitrate nutrition of plants. *K. Lantbrukshögskolans Ann.* **13**, 1.

Burström, H. (1947). A preliminary study on mineral nutrition and cell elongation of roots. *K. fysiog. Sällsk. Lund Förh.* **17**, 1.

Buston, H. W., Kasinathan, S. & Wylie, S. M. (1938). The nitrogen requirements of *Nematospora gossypii* in synthetic media. *Ann. Bot., Lond.*, N.S. **2**, 373.

Butkevich, V. S. & Kolesnikova, N. A. (1941). Formation of ammonia in the process of fixation of molecular nitrogen by *Azotobacter*. *C.R. Acad. Sci. U.R.S.S.* **33**, 66.

Butler, J. A. V. (1946). Life and the second law of thermodynamics. *Nature, Lond.*, **158**, 153.

Bychkov, S. M. (1939). Amino-nitrogen transfer by α-monoaminocarboxylic acids carrying a second acid group: Cysteic acid and phosphoserine. *Biokhim.* **4**, 189 (Russian) (quoted from *Chem. Abstr.* **34**, 1694).

Chailakhyan, M. C. (1944). Flowering in different plant species as a response to nitrogenous food. *C.R. Acad. Sci. U.R.S.S.* **47**, 146.

Chailakhyan, M. C. & Lukovnikov, E. K. (1941). Development of plants growing under different light conditions as affected by mineral nutrition. *C.R. Acad. Sci. U.R.S.S.* **32**, 152.

Chailakhyan, M. C. & Megrbyan, A. A. (1944). Effect of day-length upon the formation of root nodules on the roots of leguminous plants. *C.R. Acad. Sci. U.R.S.S.* **47**, 439.

Chapman, H. D. (1943). Failure of vetch to excrete nitrogen from the nodules when grown in association with nitrogen-deficient citrus seedlings. *J. Amer. Soc. Agron.* **35**, 635.

Chapman, H. D., Brown, S. M. & Liebig, G. F. (1943). Some effects on citrus fruit quality of nitrogen, phosphorus and potassium. *Calif. Citrogr.* **28**, 198, 211, 230, 246.

Chargaff, E. & Sprinson, D. B. (1943). The mechanism of deamination of serine by *Bacterium coli*. *J. biol. Chem.* **148**, 249.

Chatterjee, R. (1943). On the histological distribution of alkaloids in the Himalayan *Berberes*. *J. Amer. pharm. Ass.* **32**, 1.

Chaudhuri, H. (1940). Nitrogen fixation in the rice-field soils of Bengal. *Nature, Lond.*, **145**, 936.

Chibnall, A. C. (1939). *Protein Metabolism in the Plant.* New Haven, Conn.

Chibnall, A. C. (1942). Aminoacid analysis and the structure of proteins. *Proc. roy. Soc.* B, **131**, 136.

Chibnall, A. C. (1946). The contribution of the analytical chemist to the problem of protein structure. *J. int. Soc. Leath. Chem.* **30**, 1.

Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943*a*). The total nitrogen content of egg albumin and other proteins. *Biochem. J.* **37**, 354.

Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943*b*). The dicarboxylic and basic amino-acids of edestin, egg albumin and β-lactoglobulin. *Biochem. J.* **37**, 372.

Cholodny, N. (1926). *Die Eisenbakterien.* Jena.

Chu, S. P. (1942). The influence of the mineral composition of the medium on the growth of planktonic algae. Part I. Methods and culture media. *J. Ecol.* **30**, 284.

Ciferri, R. (1946). Studi sul biochimismo del tabacco. III. Influenza del nucleo piridinico sulla formazione degli alcaloidi del tabacco. *Boll. Soc. ital. Biol. sper.* **21**, 203.

Ciferri, R. & Pratesi, P. (1946). Studi sul biochimismo del tabacco. II. Effetto dell' acido nicotinico e derivati sul metabolismo e sul contenuto in alcaloidi. *Boll. Soc. ital. Biol. sper.* **21**, 197.

Clark, H. E. (1936). The effect of ammonium and of nitrate nitrogen on the composition of the tomato plant. *Plant Physiol.* **11**, 5.

Clements, H. F. (1940). Integration of climatic and physiologic factors with reference to the production of sugar cane. *Hawaii. Plant. Rec.* **44**, 201.

Cohen, P. P. (1939*a*). Microdetermination of glutamic acid. *Biochem. J.* **33**, 551.

Cohen, P. P. (1939*b*). Transamination in pigeon breast muscle. *Biochem. J.* **33**, 1478.

Cohen, P. P. (1940). Transamination with purified enzyme preparations (transaminases). *J. biol. Chem.* **136**, 565.

Cohen, P. P. (1945). The metabolism of protein and amino acids. *Ann. Rev. Biochem.* **14**, 357.

Cohen, P. P. & Hayano, M. (1946*a*). The conversion of citrulline to arginine (transamination) by tissue slices and homogenates. *J. biol. Chem.* **166**, 239.

Cohen, P. P. & Hayano, M. (1946*b*). Urea synthesis by liver homogenates. *J. biol. Chem.* **166**, 251.

Cohen, P. P. & Hayano, M. (1947). The conversion of ornithine to citrulline by rat liver homogenates. *J. biol. Chem.* **170**, 687.

Cohen, P. P. & Hekhuis, G. L. (1941). Rate of transamination in normal tissues. *J. biol. Chem.* **140**, 711.

Cohen, P. P. & McGilvery, R. W. (1947*a*). Peptide bond synthesis. II. The formation of *p*-aminohippuric acid by liver homogenates. *J. biol. Chem.* **169**, 119.

Cohen, P. P. & McGilvery, R. W. (1947*b*). Peptide bond synthesis. III. On the mechanism of *p*-aminohippuric acid synthesis. *J. biol. Chem.* **171**, 121.

Collier, H. B. (1940). The problem of plastein formation. II. The chemical changes involved in plastein formation by papain and by pepsin. *Canad. J. Res.* B, **18**, 272.

Common, R. H. (1945). Application of the chemiluminescence test for haematin to plant tissues. *Nature, Lond.*, **155**, 604.

Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). Qualitative analysis of proteins: a partition chromatographic method using paper. *Biochem. J.* **38**, 224.

Craig, L. C. & Jacobs, W. A. (1943*a*). The veratrine alkaloids. XIV. The correlation of the veratrine alkaloids with the *Solanum* alkaloids. *Science*, **97**, 122.

Craig, L. C. & Jacobs, W. A. (1943*b*). The veratrine alkaloids. XX. Further correlations in the veratrine group. The relationship between the veratrine bases and solanidine. *J. biol. Chem.* **149**, 451.

Cromwell, B. T. (1943*a*). Studies on the synthesis of hyoscyamine in *Atropa belladonna* L. and *Datura stramonium* L. *Biochem. J.* **37**, 717.

Cromwell, B. T. (1943*b*). The role of putrescine in the synthesis of hyoscyamine. *Biochem. J.* **37**, 722.

Cruickshank, D. H. & Wood, J. G. (1945). The metabolism of starving leaves. VI. Nitrogen balance sheet and changes in organic acid content during starvation of oat leaves. *Aust. J. exp. Biol. med. Sci.* **23**, 243.

Curtis, L. C. (1944). The exudation of glutamine from lawn grass. *Plant Physiol.* **19**, 1.

Damodaran, M. & Nair, K. R. (1938). Glutamic acid dehydrogenase from germinating seeds. *Biochem. J.* **32**, 1065.

Daniel, L. & Potel, E. (1925). Greffes de Douce-Amère sur racines de Belladonne. *C.R. Acad. Sci., Paris*, **181**, 357.

Danilevski, A. Y. (1886). *Organoplastic Forces of the Organism.* Kharkov (quoted from Blagoveshchenski (1940)) (Russian).

Dawson, R. F. (1939). Influence of certain amino acids and of nicotinic acid upon the nicotine content of tobacco leaves. *Plant Physiol.* **14**, 479.

Dawson, R. F. (1940). Metabolism of nicotine monohydrochloride in excised tobacco leaves. *Amer. J. Bot.* **27**, 190.

Dawson, R. F. (1941). The localisation of the nicotine synthetic mechanism in the tobacco plant. *Science*, **94**, 396.

Dawson, R. F. (1942*a*). Accumulation of nicotine in reciprocal grafts of tomato and tobacco. *Amer. J. Bot.* **29**, 66.

Dawson, R. F. (1942*b*). Nicotine synthesis in excised tobacco roots. *Amer. J. Bot.* **29**, 813.

Dawson, R. F. (1944*a*). Translocation of hyoscyamine in *Datura stramonium* L. *Amer. J. Bot.* **31**, 95 (abstract only).

Dawson, R. F. (1944*b*). Accumulation of anabasine in reciprocal grafts of *Nicotiana* and tomato. *Amer. J. Bot.* **31**, 351.

Dawson, R. F. (1945*a*). An experimental analysis of alkaloid production in *Nicotiana*: The origin of nornicotine. *Amer. J. Bot.* **32**, 416.

Dawson, R. F. (1945*b*). On the biogenesis of nornicotine and anabasine. *J. Amer. chem. Soc.* **67**, 503.

Dawson, R. F. (1946). Development of some recent concepts in the physiological chemistry of the tobacco alkaloids. *Plant Physiol.* **21**, 115.

De Graeve, P. (1937). Évolution de l'azote purique au cours de la germination. *C.R. Acad. Sci., Paris*, **204**, 798.

Demidenko, T. T. & Timofeyeva, E. F. (1937*a*). *Azotobacter* as a source of nitrogenous nourishment for the higher plants. *C.R. Acad. Sci. U.R.S.S.* **14**, 205.

Demidenko, T. T. & Timofeyeva, E. F. (1937*b*). The influence of nodule bacteria and the *Azotobacter* on the yield of leguminous and cereal plants, sown together. *C.R. Acad. Sci. U.R.S.S.* **14**, 231.

De Tempe, J. (1945). Alkaloïdvorming door *Claviceps purpurea* (Fr.) Tul. in saprophytische cultuur. Thesis, Amsterdam.

Dmitriev, K. A. (1939*a*). Effect of microelements on the production of seed and hay by red clover. *Dokl. Vsesoyuz. Akad. S.-Kh. Nauk imeni V. I. Lenina* no. 10, p. 16 (Russian).

Dmitriev, K. A. (1939*b*). The action of microelements on the development and yield of red clover on limed podsolized soils. *Pochvovedenie* (*Pedology*), no. 4, p. 114 (Russian).

Donen, I. (1937). Studies in deciduous fruit. V. Preliminary observations on the relationship between nitrogenous metabolism and internal breakdown of Kelsey plums in cold store. *Trans. roy. Soc. S. Afr.* **25**, 83.

DONEN, I. (1938). N metabolism of the Kelsey plum. *Biochem. J.* **32**, 366.

DUBOIS, K. P. & ERWAY, W. F. (1946). Studies on the mechanism of action of thiourea and related compounds. II. Inhibition of oxidative enzymes and oxidations catalysed by copper. *J. biol. Chem.* **165**, 711.

DUGGAR, B. M. & DAVIS, A. R. (1916). Studies in the physiology of the fungi. I. Nitrogen fixation. *Ann. Mo. bot. Gdn*, **3**, 413.

DU VIGNEAUD, V. & IRISH, O. J. (1938). The rôle of the acetyl derivative as an intermediary stage in the biological synthesis of amino acids from keto acids. *J. biol. Chem.* **122**, 349.

DU VIGNEAUD, V., SIMMONDS, S., CHANDLER, J. P. & COHN, M. (1946). A further investigation of the rôle of betaine in transmethylation reactions *in vivo*. *J. biol. Chem.* **165**, 639.

EATON, S. V. (1941). Influence of sulphur deficiency on metabolism of the sunflower. *Bot. Gaz.* **102**, 536.

EBERSOLE, E. R., GUTTENTAG, C. & WILSON, P. W. (1943). Nature of carbon monoxide inhibition of biological nitrogen fixation. *Arch. Biochem.* **3**, 399.

ECHEVIN, R. & BRUNEL, A. (1937*a*). Sur le métabolisme azoté au cours de la germination du Lupin (*Lupinus albus* L.). *C.R. Acad. Sci., Paris*, **204**, 881.

ECHEVIN, R. & BRUNEL, A. (1937*b*). Uréides et urée libre, dégradation des purines chez le *Soja hispida* Mnch. *C.R. Acad. Sci., Paris*, **205**, 294.

ECHEVIN, R. & BRUNEL, A. (1939). L'utilisation des uréides glyoxyliques par le *Soja*. *C.R. Acad. Sci., Paris*, **208**, 826.

EDLBACHER, S. & GRAUER, H. (1944*a*). Zur Kenntnis des Abbaues der Aminosäuren im tierischen Organismus. I. *l*-Alanin. *Helv. chim. Acta*, **27**, 151.

EDLBACHER, S. & GRAUER, H. (1944*b*). Zur Kenntnis des Abbaues der Aminosäuren im tierischen Organismus. II. Über die Spezifität der '*l*-Aminosäure-oxydase'. *Helv. chim. Acta*, **27**, 928.

EDLBACHER, S. & HEITZ, F. (1942). Zur Kenntnis des intermediären Stoffwechsels des Histidins. 7. Mitteilung. Über das Verhalten von Urocaninsäure im Tierkörper. *Hoppe-Seyl. Z.* **276**, 117.

EDLBACHER, S. & VIOLLIER, G. (1943). Die Beeinflussung des Histidinstoffwechsels durch Vitaminmangel und verschiedene Ernährungsweise. *Helv. chim. Acta*, **26**, 1978.

EDLBACHER, S. & VON BIDDER, H. (1942). Zur Kenntnis des intermediären Stoffwechsels des Histidins. 5. Mitteilung. *Hoppe-Seyl. Z.* **273**, 163.

EISENMENGER, W. S. (1933). The distribution of nitrogen in tobacco when the supplies of nitrogen and of light are varied during the growing period. *J. agric. Res.* **46**, 255.

ENDERS, C. & GLAWE, R. (1942). Über den Nicotinabbau im Tabakblatt. *Biochem. Z.* **312**, 277.

ENDRES, G. (1936). Beiträge zur Kenntnis der biologischen Bindung des Luftstickstoffes. *Angew. Chem.* **49**, 560.

ENGEL, H. & ROBERG, M. (1938). Die Stickstoffausscheidung der Wurzelknöllchen. *Ber. dtsch. bot. Ges.* **56**, 337.

EPPS, H. M. R. (1945). Studies on bacterial aminoacid decarboxylases. 4. *l*-(+)-histidine decarboxylase from *Cl. welchii* type A. *Biochem. J.* **39**, 42.

EVANS, E. A. & SLOTIN, L. (1940). The rôle of carbon dioxide in the synthesis of urea in rat liver slices. *J. biol. Chem.* **136**, 805.

EVANS, E. A., VENNESLAND, B. & SLOTIN, L. (1943). The mechanism of carbon dioxide fixation in cell-free extracts of pigeon liver. *J. biol. Chem.* **147**, 771.

FAGAN, T. W. & ASHTON, W. M. (1938). The effect of partial field-drying and artificial drying on the chemical composition of grass. *Welsh J. Agric.* **14**, 160.

FARRIES, E. M. H. & BELL, A. F. (1930). On the metabolism of Nematospora gossypii and related fungi, with special reference to the source of nitrogen. *Ann. Bot., Lond.*, **44**, 424.

FELIX, K. & NAKA, S. (1940). Die Wirkung von Leber- und Nierenschnitten auf *l*-(+)-Lysin, *l*-(+)-Glutaminsäure und *l*-(+)-Asparaginsäure. *Hoppe-Seyl. Z.* **264**, 123.

FERDMAN, D. L., FRENKEL, S. R. & SILAKOVA, A. I. (1942). Glutamine in the tissues of animal organisms. *Biokhim.* **7**, 43 (Russian) (quoted from *Chem. Abstr.* **37**, 4451).

FIFE, J. M. & FERGUSON, W. C. (1941). The effect of high concentrations of carbon dioxide on the glutamine and asparagine metabolism of the sugar beet. *Plant Physiol.* **16**, 677.

FINCH, A. H. (1944). Fertilizing desert grapefruit. *Calif. Citr.* **30**, 34.

FINCH, A. H., JONES, W. W. & VAN HORN, C. W. (1945). The influence of nitrogen nutrition upon the ascorbic acid content of several vegetable crops. *Proc. Amer. Soc. hort. Sci.* **46**, 314.

FISCHER, E. & GÄUMANN, E. A. (1929). *Biologie der Pflanzenbewohnenden Pilze.* Jena.

FISCHER, H. (1936). Untersuchungen über die Stickstoffwanderung in der höheren Pflanze. *Z. Bot.* **30**, 449.

FODOR, A. & REIFENBERG, A. (1927). The enzymic production of volatile products from nicotine under the influence of tobacco-leaf extracts. (A reply to A. Faitelowitz.) *Biochem. J.* **21**, 765.

FOLKERS, K., KONUISZY, F. & SHAVEL, J. (1944). *Erythrina* alkaloids. XIV. Isolation and characterisation of erysothiovine and erysothiopine, new alkaloids containing sulfur. *J. Amer. chem. Soc.* **66**, 1083.

FOSSE, R. (1939). Uréogenase et métabolisme de l'azote purique chez les végétaux. *C.R. Acad. Sci., Paris*, **208**, 865.

Foster, G. L., Schoenheimer, R. & Rittenberg, D. (1939). Studies in protein metabolism. V. The utilisation of ammonia for amino-acid and creatine formation in animals. *J. biol. Chem.* **127**, 319.

Francis, W. D. (1925). A contribution to the theory of the relationship of iron to the origin of life. *Proc. roy. Soc. Qd*, **37**, 98.

Francis, W. D. (1932). The production of protein by inorganic material: Evidence suggestive of the generation of life. *Proc. roy. Soc. Qd*, **44**, 23.

Francis, W. D. (1933). *The Origin, Classification and Organic Relationships of the Protein Produced by Inorganic Ferruginous Material.* Brisbane.

Francis, W. D. (1934). *The Mechanism of the Production of Protein from Inorganic Material by Iron: The Relationship of the Iron Bacterium* Leptothrix *to Nuclear Chromosomes.* Brisbane.

Francis, W. D. (1935). *Iron as the Original Basis of Protoplasm: The Generation of Life in Space and Time.* Brisbane.

Frankenburg, W. G. (1946). Chemical changes in the harvested tobacco leaf. Part I. Chemical and enzymic conversions during the curing process. *Adv. Enzymol.* **6**, 309.

Franzen, H. & Helwert, F. (1922). Über die chemischen Bestandteile grüner Pflanzen. 20. Mitteilung. Über die Säuren der Kirschen (*Prunus avium*). *Hoppe-Seyl. Z.* **122**, 46.

Franzen, H. & Helwert, F. (1923). Über die chemischen Bestandteile grüner Pflanzen. 26. Mitteilung. Kritisches über das Vorkommen der Weinsäure in den Pflanzen. *Biochem. Z.* **136**, 291.

Franzen, H. & Stern, E. (1922). Über die chemischen Bestandteile grüner Pflanzen. 19. Mitteilung. Über das Vorkommen von Milchsäure und Bernsteinsäure in den Blättern der Himbeere (*Rubus idaeus*). *Hoppe-Seyl. Z.* **121**, 195.

Frazer, H. L. (1942). The occurrence of endodermis in leguminous root nodules and its effect upon nodule function. *Proc. roy. Soc. Edinb.* **61**, 328.

Frey-Wyssling, A. (1938). Über die Herkunft der sekundären Pflanzenstoffe. *Naturwissenschaften*, **26**, 624.

Frey-Wyssling, A. (1942). Zur Physiologie der pflanzlichen Glukoside. *Naturwissenschaften*, **30**, 500.

Frey-Wyssling, A. & Blank, F. (1940). Growth of protoplasm and nitrogen migration in the coleoptile of *Zea mays*. *Nature, Lond.*, **145**, 974.

Fritsch, F. E. & De, P. K. (1938). Nitrogen fixation by blue-green algae. *Nature, Lond.*, **142**, 878.

Gale, E. F. & Epps, H. M. R. (1944*a*). Studies on bacterial aminoacid decarboxylases. 1. *l*-(+)-lysine decarboxylase. *Biochem. J.* **38**, 232.

Gale, E. F. & Epps, H. M. R. (1944*b*). Studies on bacterial aminoacid decarboxylases. 3. Distribution and preparation of codecarboxylase. *Biochem. J.* **38**, 250.

Gale, E. F. & Tomlinson, H. M. R. (1946). Co-aminopherase, co-decarboxylase and pyridoxal. *Nature, Lond.*, **158**, 103.

Garner, W. W., Bacon, C. W., Bowling, J. D. & Brown, D. E. (1934). The nitrogen nutrition of tobacco. *Tech. Bull. U.S. Dep. Agric.* no. 414.

Gilbert, S. G., Sell, H. M. & Drosdoff, M. (1946). The effect of copper deficiency on the nitrogen metabolism and oil synthesis of the tung tree. *Plant Physiol.* **21**, 290.

Gilbert, S. G. & Shive, G. W. (1945). The importance of oxygen in the nutrient substrate for plants. Relation of the nitrate ion to respiration. *Soil Sci.* **59**, 453.

Gollub, M. C. & Vennesland, B. (1947). Fixation of carbon dioxide by a plant oxalacetate carboxylase. *J. biol. Chem.* **169**, 233.

Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1943). The amino-acid composition of tyrocidine. *Biochem. J.* **37**, 313.

Gorham, P. R. (1945). Growth factor studies with *Spirodela polyrhiza* (L.) Schleid. *Amer. J. Bot.* **32**, 496.

Gornall, A. G. & Hunter, A. (1943). The synthesis of urea in the liver, with special reference to citrulline as an intermediary in the ornithine cycle. *J. biol. Chem.* **147**, 593.

Grafe, V. & Linsbauer, K. (1906). Über die wechselseitige Beeinflussung von *Nicotiana tabacum* und *N. affinis* bei der Pfropfung. *Ber. dtsch. bot. ges.* **24**, 366.

Granick, S. (1938*a*). Chloroplast nitrogen of some higher plants. *Amer. J. Bot.* **25**, 561.

Granick, S. (1938*b*). Urease distribution in *Soja max*. *Plant Physiol.* **13**, 29.

Granick, S. & Gilder, H. (1946). The porphyrin requirements of *Haemophilus influenzae* and some functions of the vinyl and propionic acid side chains of heme. *J. gen. Physiol.* **30**, 1.

Greathouse, G. A. (1939). Alkaloids from *Sanguinaria canadensis* and their influence on growth of *Phymatotrichum omnivorum*. *Plant Physiol.* **14**, 377.

Greathouse, G. A. & Watkins, G. M. (1938). Berberine as a factor in the resistance of *Mahonia trifoliata* and *M. swaseyi* to *Phymatotrichum* root rot. *Amer. J. Bot.* **25**, 743.

Grebinski, S. O. (1941). Changes in the chemical composition of cultivated plants when grown in the mountains (beet, peas, *Nicotiana rustica*, barley). *C.R. Acad. Sci. U.R.S.S.* **32**, 285.

Green, D. E., Leloir, L. F. & Nocito, V. (1945). Transaminases. *J. biol. Chem.* **161**, 559.

Gregory, F. G. & Sen, P. K. (1937). Physiological studies in plant nutrition. VI. The relation of respiration rate to the carbohydrate and nitrogen metabolism of the barley leaf as determined by nitrogen and potassium deficiency. *Ann. Bot., Lond.*, N.S. **1**, 521.

GRIFFITH, R. B. (1943). Effect of N on anther colour in Kentucky bluegrass. *J. Amer. Soc. Agron.* **35**, 348.

GRODZENSKI, D. E. (1940). Glycolysis and transamination. II. Effect of *l*-(+)-glutamic acid on glycolysis of brain tissue. *Byull. Eksp. Biol. Med.* **10**, 484 (Russian).

GRONER, M. G. (1936). Amino nitrogen and reducing sugars of green and chlorophyll-deficient types in maize. *Amer. J. Bot.* **23**, 453.

GRONER, M. G. (1939). Sugar excretion in *Impatiens sultani*. *Amer. J. Bot.* **26**, 464.

GUEST, P. (1943). A comparison of certain chemical constituents of green and chlorotic *Macadamia* leaves. *Proc. Amer. Soc. hort. Sci.* **42**, 104.

GULICK, A. (1938). What are the genes? I. The genetic and evolutionary picture. II. The physicochemical picture; conclusions. *Quart. Rev. Biol.* **13**, 1, 140.

GUNSALUS, I. C., UMBREIT, W. W., BELLAMY, W. D. & FOUST, C. E. (1945). Some properties of synthetic codecarboxylase. *J. biol. Chem.* **161**, 743.

HAC, L. R. & SNELL, E. E. (1945). The microbiological determination of amino acids. III. Assay of aspartic acid with *Leuconostoc mesenteroides*. *J. biol. Chem.* **159**, 291.

HAC, L. R., SNELL, E. E. & WILLIAMS, R. J. (1945). The microbiological determination of amino acids. II. Assay and utilisation of glutamic acid and glutamine by *Lactobacillus arabinosus*. *J. biol. Chem.* **159**, 273.

HAHN, G., BÄRWALD, L., SCHALES, O. & WERNER, H. (1935). Synthese von Tetrahydroharman (4-Carbolin)-Derivaten unter physiologischen Bedingungen. II. Mitteilung. *Liebigs Ann.* **520**, 107.

HAHN, G. & WERNER, H. (1935). Synthese von Tetrahydro-harman (4-Carbolin)-Systemen unter physiologischen Bedingungen. III. Mitteilung. Synthese des Yohimbin-Gerüstes. *Liebigs Ann.* **520**, 123.

HAINES, W. B. (1946). Manuring *Hevea*. Part IV. Conspectus of experimental improvements achieved in mature stands at the end of ten years, with a special note on seed production. *Emp. J. exp. Agric.* **14**, 182.

HALDANE, J. B. S. (1937). The biochemistry of the individual. In *Perspectives of Biochemistry*. Cambridge.

HALL, J. A. (1937). A system of structural relationships in phytochemistry. *Chem. Rev.* **20**, 305.

HAMADA, M. (1940). Physiologisch-morphologische Studien über *Armillaria mellea* (Vahl) Quél., mit besonderer Rücksicht auf die Oxalsäurebildung. Ein Nachtrag zur Mykorrhiza von *Galeola septentrionalis* Rchb. f. *Jap. J. Bot.* **10**, 387.

HAMILTON, P. B. (1945). Glutamine: a major constituent of free α-amino acids in animal tissues and blood plasma. *J. biol. Chem.* **158**, 397.

HAMNER, K. C. (1936). Effects of nitrogen supply on rates of photosynthesis and respiration in plants. *Bot. Gaz.* **97**, 744.

HAMNER, K. C. & NIGHTINGALE, G. T. (1946). Ascorbic acid content of pineapples as correlated with environmental factors and plant composition. *Food Res.* **11**, 535.

HAMPE, W. (1865). Über die Assimilation von Harnstoff und Ammoniak durch Pflanzen. *Landw. Vers Sta.* **7**, 308.

HANSON, E. A. (1939). Some properties of the chlorophyll in relation to its biological function. *Rec. Trav. bot. néerl.* **36**, 180.

HANSON, E. A. (1941). A note on the metabolism of chloroplast protein. *Aust. J. exp. Biol. med. Sci.* **19**, 157.

HANSON, E. A., BARRIEN, B. S. & WOOD, J. G. (1941). Relations between protein-nitrogen, protein-sulphur and chlorophyll in leaves of Sudan grass. *Aust. J. exp. Biol. med. Sci.* **19**, 231.

HANSTEEN, B. (1899). Über Eiweisssynthese in grünen Phanerogamen. *Jb. wiss. Bot.* **33**, 417.

HARMER, P. M. & SHERMAN, G. D. (1943). The effect of manganese deficiency on the synthesis of ascorbic acid (vitamin C) in foliage of plants. *Proc. Soil Sci. Soc. Amer.* **8**, 346.

HARTT, C. E. (1934). Some effects of potassium upon the amounts of protein and amino forms of nitrogen, sugars, and enzyme activity of sugar cane. *Plant Physiol.* **9**, 453.

HASEMAN, L. (1946). Influence of soil minerals on insects. *J. econ. Ent.* **39**, 8.

HAWORTH, W. N. (1947). Carbohydrate components of biologically active materials. *J. chem. Soc.* p. 582.

HELGESON, E. A. & HARRIS, R. H. (1941). Quality of Thatcher wheat grown in soilless culture. *Bi-m. Bull. N. Dak. agric. exp. Sta.* no. 5, p. 3.

HENRIQUES, V. & GJALDBÄK, I. K. (1911). Untersuchungen über die Plasteinbildung. *Hoppe-Seyl. Z.* **71**, 485.

HENRY, T. A. (1939). *The Plant Alkaloids*, 3rd ed. London.

HERBST, R. M. & RITTENBERG, D. (1943). The transamination reaction. The mechanism of the reaction between *alpha* keto acids and *alpha* amino acids. *J. organ. Chem.* **8**, 380.

HERBST, R. M. & SHEMIN, D. (1943). The synthesis of peptides by transamination. *J. biol. Chem.* **147**, 541.

HEVESY, G., LINDERSTRØM-LANG, K., KESTON, A. S. & OLSEN, C. (1940). Exchange of nitrogen atoms in the leaves of the sunflower. *C.R. Lab. Carlsberg*, **23**, 213.

HICKS, C. S. & LE MESSURIER, H. (1935). Preliminary observations on the chemistry and pharmacology of the alkaloids of *Duboisia hopwoodii*. *Aust. J. exp. Biol. med. Sci.* **13**, 175.

Hicks, C. S. & Sinclair, D. A. (1947). Alkaloids of *Nicotiana excelsior* and *Duboisia hopwoodii*. *Aust. J. exp. Biol. med. Sci.* **25**, 191.

Hida, T. (1941). Über den Einfluss des Natriumfluorids und der anorganischen Stickstoffquellen auf der Stoffwechsel von *Aspergillus niger*, mit besonderer Berücksichtigung der Bildung von Brenztraubensäure und Dimethylbrenztraubensäure. *J. Shanghai Sci. Inst.* Sect. IV, **5**, 199.

Hilgeman, R. H. (1941). Nitrogen uptake by grapefruit trees in the Salt River valley. *Proc. Amer. Soc. hort. Sci.* **39**, 119.

Hills, K. L. (1945). Changes in the morphine and dry matter content of the opium poppy (*Papaver somniferum*) during the maturation period. *J. Coun. sci. industr. Res. Aust.* **18**, 286.

Hills, K. L. & Rodwell, C. (1946). The distribution and nature of the alkaloids in developing seedlings of *Duboisia myoporoides* and *Duboisia leichhardtii*. *J. Coun. sci. industr. Res. Aust.* **19**, 295.

Hills, K. L., Trautner, E. M. & Rodwell, C. N. (1945*a*). A preliminary report upon variation in the nature and quantity of the main alkaloids in *Duboisia myoporoides* and *Duboisia leichhardtii*. *J. Coun. sci. industr. Res. Aust.* **18**, 234.

Hills, K. L., Trautner, E. M. & Rodwell, C. N. (1945*b*). The presence of hyoscine in tomato scions on *Duboisia* rootstocks. *Aust. J. Sci.* **8**, 20.

Hoener, I. R. & De Turk, E. E. (1938). The absorption and utilisation of nitrate nitrogen during vegetative growth by Illinois high protein and Illinois low protein corn. *J. Amer. Soc. Agron.* **30**, 232.

Horn, M. J. & Jones, D. B. (1940). Isolation of a crystalline selenium-containing organic compound from plant material. *J. Amer. chem. Soc.* **62**, 234.

Horner, C. K. & Allison, F. E. (1944). Utilisation of fixed nitrogen by *Azotobacter* and influence on nitrogen fixation. *J. Bact.* **47**, 1.

Horner, C. K., Burk, D., Allison, F. E. & Sherman, M. S. (1942). Nitrogen fixation by *Azotobacter* as influenced by molybdenum and vanadium. *J. agric. Res.* **65**, 173.

Horowitz, B. (1933). Kilka spostrzeżeń nad ocienianiem tytoniu Connecticut Havanna. (Observations on shading the tobacco variety Connecticut Havana.) *Roczn. Nauk. Roln. i Les.* **30**, 1.

Horowitz, B. (1942). Nicotine products and their importance as insecticides. *Aust. J. Sci.* **4**, 179.

Horowitz, B. (1947). Personal communication.

Horowitz, N. H. (1945). On the evolution of biochemical syntheses. *Proc. nat. Acad. Sci., Wash.*, **31**, 153.

Horowitz, N. H. (1946). The isolation and identification of a natural precursor of choline. *J. biol. Chem.* **162**, 413.

Horowitz, N. H. (1947). Methionine synthesis in *Neurospora*. The isolation of cystathionine. *J. biol. Chem.* **171**, 255.

Horowitz, N. H., Bonner, D., Mitchell, H. K., Tatum, E. L. & Beadle, G. W. (1945). Genic control of biochemical reactions in *Neurospora*. *Amer. Nat.* **79**, 304.

Hulme, A. C. (1936). Metabolism of nitrogen in apple fruits. *Dept. Sci. Ind. Res.* (Brit.), *Rep. Food Invest. Bd*, p. 126.

Hulme, A. C. (1946). The protein of fruits. *Nature, Lond.*, **158**, 58, 588.

Hurwitz, C. & Wilson, P. W. (1940). Direct estimation of biological nitrogen fixation. A gasometric method. *Industr. Engng Chem.* (Anal. ed.), **12**, 31.

Irving, A. A. & Hankinson, R. (1908). The presence of a nitrate reducing enzyme in green plants. *Biochem. J.* **3**, 87.

Ivanov, N. N. (1926). The influence of geographic factors on the chemical composition of plants. *Ann. St. Inst. Exp. Agron.* **4**, 23 (Russian) (quoted from *Chem. Abstr.* **22**, 1380).

Ivanov, N. N. & Knyaginichev, M. A. (1936). *Biochemistry of Cultivated Plants.* 1. *Cereals.* Moscow-Leningrad (Russian).

Ivanov, N. N. & Krupkina, F. A. (1929). Über die Stickstoffausscheidung der Hefe während der Gärung. *Biochem. Z.* **212**, 255.

James, W. O. (1946*a*). Demonstration of alkaloids in solanaceous meristems. *Nature, Lond.*, **158**, 377.

James, W. O. (1946*b*). Biosynthesis of the *Belladonna* alkaloids. *Nature, Lond.*, **158**, 654.

Jensen, H. L. (1945). Nitrogen fixation in leguminous plants. VI. Further observations on the effect of molybdenum on symbiotic nitrogen fixation. *Proc. Linn. Soc. N.S.W.* **70**, 203.

Jensen, H. L. & Betty, R. C. (1943). Nitrogen fixation in leguminous plants. III. The importance of molybdenum in symbiotic nitrogen fixation. *Proc. Linn. Soc. N.S.W.* **68**, 1.

Jensen, H. L. & Frith, D. (1944). Production of nitrate from roots and root nodules of lucerne and subterranean clover. *Proc. Linn. Soc. N.S.W.* **69**, 210.

Jensen, H. L. & Spencer, D. (1947). The influence of molybdenum and vanadium on nitrogen fixation by *Clostridium butyricum* and related organisms. *Proc. Linn. Soc. N.S.W.* **72**, 73.

Jones, W. W., Bitters, W. P. & Finch, A. H. (1944). The relation of nitrogen absorption to nitrogen content of fruit and leaves in citrus. *Proc. Amer. Soc. hort. Sci.* **45**, 1.

Kabos, W. J. (1936). Beitrag zur Kenntnis des *N*-Stoffwechsels von *Sinapis alba*, besonders in Bezug auf das Licht. *Rec. trav. bot. néerl.* **33**, 447.

Kamen, M. D. (1947*a*). *Radioactive Tracers in Biology.* New York.

Kamen, M. D. (1947*b*). Use of isotopes in biochemical research: Fundamental aspects. *Ann. Rev. Biochem.* **16**, 631.

Kaplanski, S. & Shmerling, Z. (1945). Synthesis of amino acids in tissues. Part V. Synthesis of amino acids from dicarboxylic acids in liver and kidney slices. *Biokhim.* **10**, 295 (Russian).

Kappen, H. & Wienhues, W. (1942). Über die Aufnahme des Stickstoffs der Ammoniumsalze und der Nitrate durch Keimpflanzen. Mitteilung I. *Bodenk. Pflanz.* **27**, 311.

Karrer, P. (1938). *Organic Chemistry.* Amsterdam.

Karyagina, M. K. (1939). Formation and breakdown of amino acids by intermolecular transfer of amino groups. VI. Metabolism of *l*-(−)-aspartic acid in different animal tissues. *Biokhim.* **4**, 168 (Russian) (quoted from *Chem. Abstr.* **34**, 1693).

Keilin, D. & Smith, J. D. (1947). Haemoglobin and nitrogen fixation in the root nodules of leguminous plants. *Nature, Lond.*, **159**, 692.

Keilin, D. & Wang, Y. L. (1945). Haemoglobin in the root nodules of leguminous plants. *Nature, Lond.*, **155**, 227.

Keirstead, L. G. (1945). Relation of carotene and crude protein content of grasses. *J. Amer. Soc. Agron.* **37**, 239.

Kerkis, J. J. & Pigulevskaya, N. N. (1941). Interaction between *Lycopersicum esculentum* and *Datura stramonium* in the case of grafting. *C.R. Acad. Sci. U.R.S.S.* **32**, 505.

Ketchum, B. H. (1939). The absorption of phosphate and nitrate by illuminated cultures of *Nitzschia closterium*. *Amer. J. Bot.* **26**, 399.

Kidd, F., West, C., Griffiths, D. G. & Potter, N. A. (1940). An investigation of the changes in chemical composition and respiration during the ripening and storage of Conference pears. *Ann. Bot., Lond.*, N.S. **4**, 1.

Knoop, F. & Martius, C. (1938). Über den Reaktionsmechanismus der Umaminierung von Aminosäuren (Vorläufige Mitteilung). *Hoppe-Seyl. Z.* **254**, 1.

Knoop, F. & Oesterlin, H. (1925). Über die natürliche Synthese der Aminosäuren und ihre experimentelle Reproduktion. *Hoppe-Seyl. Z.* **148**, 294.

Knudson, L. (1933). Investigations on the use of certain amino acids by green plants. *Amer. J. Bot.* **20**, 680 (abstract only).

Knyaginichev, M. I. (1939). Protein in Soviet wheats. *Bull. Acad. Sci. U.R.S.S.*, Sér. Biol. p. 880 (Russian).

Konikova, A. S., Dobbert, N. N. & Braunstein, A. E. (1947). Labilization of the α-hydrogen of amino-acids in the presence of aminopherase. *Nature, Lond.*, **159**, 67.

Korsakova, M. P. (1941). Reduction of nitrates to ammonia by facultative and obligate anaerobes. *Mikrobiologiya*, **10**, 299 (Russian).

Kostina, K. F. (1936). *The Apricot.* Suppl. 83 to *Bull. Appl. Bot. Genet. Select.* Leningrad.

Kostov, D. (1943). *Cytogenetics of the Genus* Nicotiana. Sofia (Bulgarian and English).

Kovalenko, E. I. (1934). Characteristics of some *Nicotiana* species. *Rep. on Tobacco Chem. All Union Inst. Tobacco Industr.* **5**, 15 (Russian) (quoted from Kostov (1943)).

Krampitz, L. O., Wood, H. G. & Werkman, C. H. (1943). Enzymatic fixation of carbon dioxide in oxalacetate. *J. biol. Chem.* **147**, 243.

Krayer, O. & Acheson, G. H. (1946). The pharmacology of the *Veratrum* alkaloids. *Physiol. Rev.* **26**, 383.

Krayevoy, S. Y. & Nechaev, I. (1941). Atropine transference from stock (*Datura stramonium*) to scion (*Solanum lycopersicum*). *C.R. Acad. Sci. U.R.S.S.* **31**, 69.

Krebs, H. A. (1935). Metabolism of aminoacids. IV. The synthesis of glutamine from glutamic acid and ammonia, and the enzymic hydrolysis of glutamine in animal tissues. *Biochem. J.* **29**, 1951.

Krebs, H. A. (1942). Urea formation in mammalian liver. *Biochem. J.* **36**, 758.

Krebs, H. A. (1943). The intermediary stages in the biological oxidation of carbohydrate. *Adv. Enzymol.* **3**, 191.

Krebs, H. A. & Cohen, P. P. (1939). Metabolism of α-ketoglutaric acid in animal tissues. *Biochem. J.* **33**, 1895.

Krebs, H. A. & Eggleston, L. V. (1945). Metabolism of acetoacetate in animal tissues. *Biochem. J.* **39**, 408.

Krebs, H. A. & Johnson, W. A. (1937). The rôle of citric acid in intermediate metabolism in animal tissues. *Enzymol.* **4**, 148.

Kritzmann, M. G. (1939). The enzyme system transferring the amino group of aspartic acid. *Nature, Lond.*, **143**, 603.

Kritzmann, M. G. (1947). The mechanism of formation of amino acids in surviving animal tissues from pyruvate and ammonia. *J. biol. Chem.* **167**, 77.

Kritzmann, M. G. & Melik-Sarkisyan, S. S. (1945*a*). Synthesis of amino acids in animal tissues. Part II. Rôle of dicarboxylic acids in formation of amino acids in liver slices. *Biokhim.* **10**, 1 (Russian).

Kritzmann, M. G. & Melik-Sarkisyan, S. S. (1945*b*). Synthesis of amino acids in animal tissues. Part III. Rôle of phosphate in the synthesis of amino acids. *Biokhim.* **10**, 336 (Russian).

Kritzmann, M. G. & Samarina, O. (1946). Reversible splitting of glutamic aminopherase. *Nature, Lond.*, **158**, 104.

Krzemieniewski, S. & Kovats, J. (1936). Über die Einfluss von Eisen und Molybdän auf die Stickstoffbindung durch *Azotobacter chroococcum* Beij. *Bull. int. Acad. polon. Sci.*, Classe Sci. Math. Nat. **1**, 169.

Kubo, H. (1939). Über Hämoprotein aus den Wurzelknöllchen von Leguminosen. *Acta Phytochim.* **11**, 195 (quoted from Wilson (1940)).

Kursanov, A. (1941). Untersuchung enzymatischer Prozesse in der lebenden Pflanze. *Adv. Enzymol.* **1**, 329.

Kursanov, A. & Bryushkova, K. (1940). Enzyme activity in leaves of different tiers in relation to their individual development and the general development of the plant. *Biokhim.* **5**, 188 (Russian).

Kuzin, A. & Polyakova, O. (1945). Synthesis of the peptide bond in presence of simple sugars. *Biokhim.* **10**, 146 (Russian).

Kuzmenko, A. A. & Tikhvinskaya, V. D. (1940). Inheritance of nicotine and anabasine by interspecific hybrids of *N. tabacum* L. and *N. glauca* Grah. and the interaction of these species when grafted. *Bull. Acad. Sci. U.R.S.S.*, Cl. Sci. Biol. p. 564 (Russian).

Kylin, A. (1943). The influence of trace elements on the growth of *Ulva lactuca*. *K. fysiog. Sällsk. Lund. Förh.* **13**, 185.

Kylin, H. (1943). Über die Ernährung von *Ulva lactuca*. *K. fysiog. Sällsk. Lund Förh.* **13**, 202.

La Fuze, H. H. (1937). Nutritional characteristics of certain wood-destroying fungi, *Polyporus betulinus* Fr., *Fomes pinicola* (Fr.) Cooke, and *Polystictus versicolor* Fr. *Plant Physiol.* **12**, 625.

Lampitt, L. H., Bushill, J. H., Rooke, H. S. & Jackson, E. M. (1943). Solanine; Glycoside of the potato. II. Its distribution in the potato plant. *J. Soc. chem. Ind., Lond.*, **62**, 48.

Lardy, H. A., Potter, R. L. & Elvehjem, C. A. (1947). The rôle of biotin in bicarbonate utilisation by bacteria. *J. biol. Chem.* **169**, 451.

Lascelles, J. & Still, J. L. (1946). The reduction of nitrate, nitrite and hydroxylamine by *E. coli*. *Aust. J. exp. Biol. med. Sci.* **24**, 159.

Lavrov, D. (1907). Über die Wirkung des Pepsins resp. Labferments auf konzentrierte Lösungen der Produkte der peptischen Verdauung der Eiweisskörper (Reaktion von A. Danilewski). *Hoppe-Seyl. Z.* **51**, 1.

Leach, L. D. & Davey, A. E. (1942). Reducing southern *Sclerotium* rot of sugar beets with nitrogenous fertilizers. *J. agric. Res.* **64**, 1.

Lebedev, S. I. (1940). Utilisation by hemp of the nitrogen of nodule bacteria. *Dokl. Vsesoyuz. Akad. S.-Kh. Nauk imeni V. I. Lenina*, no. 11, p. 33 (Russian).

Le Clerc, J. A. (1910). Trilocal experiments on the influence of environment on the composition of wheat. *Bull. U.S. Bur. Chem.* no. 128.

Lee, S. B., Wilson, J. B. & Wilson, P. W. (1942). Mechanism of biological nitrogen fixation. X. Hydrogenase in cell-free extracts and intact cells of *Azotobacter*. *J. biol. Chem.* **144**, 273.

Léeman, A. C. (1935). Hydrocyanic acid in grasses. *Onderstepoort J. vet. Sci.* **5**, 97.

Leeper, G. W. (1941). Manganese deficiency and accumulation of nitrates in plants. *J. Aust. Inst. agric. Sci.* **7**, 161.

Lehninger, A. L. (1946). The oxidation of higher fatty acids in heart muscle suspensions. *J. biol. Chem.* **165**, 131.

Lemoigne, M., Monguillon, P. & Desvaux, R. (1937*a*). Réduction de l'acide nitreux en hydroxylamine par les végétaux supérieurs. Rôle de l'acide ascorbique. *C.R. Acad. Sci., Paris*, **204**, 1841.

Lemoigne, M., Monguillon, P. Desvaux, R. (1937*b*). Nouveaux résultats sur la présence de composés volatils de l'hydroxylamine dans les feuilles fraîches de végétaux supérieurs. *Bull. Soc. Chim. biol., Paris*, **19**, 671.

Leonard, L. T. (1939). Bacteria associated with *Gleditsia triacanthos* L. *Trans. Third Comm. Int. Soc. Soil Sci.* A, p. 64.

Leonard, L. T. & Reed, H. R. (1930). A comparison of some nodule forming and non-nodule forming legumes for green manuring. *Soil Sci.* **30**, 231.

Leonard, M. J. K. & Burris, R. H. (1947). A survey of transaminases in plants. *J. biol. Chem.* **170**, 701.

Leonard, O. A. & Pinckard, J. A. (1946). Effect of various oxygen and carbon dioxide concentrations on cotton root development. *Plant Physiol.* **21**, 18.

Leroux, L. (1937). Présence de l'acide allantoïque dans les feuilles de *Corylus avellana*. *C.R. Acad. Sci., Paris*, **205**, 172.

Leuthardt, F. & Glasson, B. (1942*a*). Formation de glycocolle à partir de la sérine. *Helv. chim. Acta*, **25**, 245.

Leuthardt, F. & Glasson, B. (1942*b*). Über die biologische Harnstoffbildung. IV. Mitteilung. *Helv. chim. Acta*, **25**, 630.

Levitt, J. (1946). Osmotic pressure determinations with isolated protoplasmic proteins. *Plant Physiol.* **21**, 562.

Lichstein, H. C. & Cohen, P. P. (1945). Transamination in bacteria. *J. biol. Chem.* **157**, 85.

Lichstein, H. C., Gunsalus, I. C. & Umbreit, W. W. (1945). Function of the vitamin B_6 group: pyridoxal phosphate (codecarboxylase) in transamination. *J. biol. Chem.* **161**, 311.

Lichstein, H. C. & Umbreit, W. W. (1947). Biotin activation of certain deaminases. *J. biol. Chem.* **170**, 423.

Lind, C. J. & Wilson, P. W. (1941). Mechanism of biological nitrogen fixation by red clover. *J. Amer. chem. Soc.* **63**, 3511.

Lind, C. J. & Wilson, P. W. (1943). Carbon monoxide inhibition of *Azotobacter* in microrespiration experiments. *J. Bact.* **45**, 219.

Lipman, F. (1946). Acetyl phosphate. *Adv. Enzymol.* **6**, 231.

Lipman, J. G. & Conybeare, A. B. (1936). Preliminary note on the inventory and balance sheet of plant nutrients in the United States. *Bull. N.J. agric. Exp. Sta.* no. 607.

Loehwing, W. F. (1937). Root interactions of plants. *Bot. Rev.* **3**, 195.

Löhnis, M. P. (1930). Can *Bacterium radicicola* assimilate nitrogen in the absence of the host plant? *Soil Sci.* **29**, 37.

Loo, S. W. (1946). Preliminary experiment on the cultivation of *Baeria chrysostoma* under sterile conditions. *Amer. J. Bot.* **33**, 382.

Ludwig, C. A. (1938). The availability of different forms of nitrogen to a green alga. *Amer. J. Bot.* **25**, 448.

Ludwig, C. A. & Allison, F. E. (1937). Experiments concerning diffusion of nitrogenous compounds from healthy legume nodules or roots. *Bot. Gaz.* **98**, 680.

Lugg, J. W. H. (1940). Tissue proteins of some cryptogams; the amide, tyrosine and tryptophan contents. *Biochem. J.* **34**, 1549.

Lugg, J. W. H. (1946). Plant proteins. *Aust. Chem. Inst. J. Proc.* **13**, 88.

Lugg, J. W. H. (1947*a*). Some fundamental problems in the amino-acid analysis of proteins. *Aust. Chem. Inst. J. Proc.* **14**, 233.

Lugg, J. W. H. (1947*b*). Personal communication.

Lugg, J. W. H. & Best, R. J. (1945). The effects of infection of tobacco plants (*Nicotiana tabacum*) with tobacco mosaic virus on some of the properties of the protein present in the leaves. *Aust. J. exp. Biol. med. Sci.* **23**, 235.

Lugg, J. W. H. & Weller, R. A. (1941). Protein metabolism in seed germination. *Biochem. J.* **35**, 1099.

Lundegårdh, H. (1939). Mangan als Katalysator der Pflanzenatmung. *Planta*, **29**, 419.

Lundegårdh, H. & Stenlid, G. (1944). On the exudation of nucleotides and flavanone from living roots. *Ark. Bot.* A, **31**, 1.

Lyman, C. M., Moseley, O., Wood, S., Butler, B. & Hale, F. (1947). Some chemical factors which influence the amino acid requirements of the lactic acid bacteria. *J. biol. Chem.* **167**, 177.

Lynen, F. (1943). Zum biologischen Abbau der Essigsäure. II. Die Wirkung von Malonsäure auf den Abbau der Essigsäure durch Hefe. *Liebigs Ann.* **554**, 40.

McClure, T. T. & Robbins, W. R. (1942). Resistance of cucumber seedlings to damping-off as related to age, season of year, and level of nitrogen nutrition. *Bot. Gaz.* **103**, 684.

McGinnis, F. W. & Taylor, G. S. (1923). The effect of respiration upon the protein percentage of wheat, oats and barley. *J. agric. Res.* **24**, 1041.

McIlwain, H. (1939). The specificity of glutamine for growth of *Streptococcus haemolyticus*. *Biochem. J.* **33**, 1942.

McIlwain, H. (1946*a*). The metabolism and functions of vitamin-like compounds. 1. Ammonia formation from glutamine by haemolytic streptococci; its reciprocal connection with glycolysis. *Biochem. J.* **40**, 67.

McIlwain, H. (1946*b*). The magnitude of microbial reactions involving vitamin-like compounds. *Nature, Lond.*, **158**, 898.

McIlwain, H., Fildes, P., Gladstone, G. P. & Knight, B. C. J. G. (1939). Glutamine and the growth of *Streptococcus haemolyticus*. *Biochem. J.* **33**, 223.

McKee, H. S. (1937). A review of recent work on the nitrogen metabolism of plants. *New Phytol.* **36**, 33, 240.

McKee, H. S. (1938). The effect of varying supplies of nitrogen on the respiration of detached barley leaves. Thesis, Oxford.

McKee, M. C. & Lobb, D. E. (1938). Formation of nitrate in detached green leaves of Swiss chard and tomato. *Plant Physiol.* **13**, 407.

McLuckie, J. (1922*a*). Studies in symbiosis. I. The mycorrhiza of *Dipodium punctatum* R.Br. *Proc. Linn. Soc. N.S.W.* **47**, 293.

McLuckie, J. (1922*b*). Studies in symbiosis. II. The apogeotropic roots of *Macrozamia spiralis* and their physiological significance. *Proc. Linn. Soc. N.S.W.* **47**, 319.

McLuckie, J. (1923*a*). Studies in symbiosis. III. Contribution to the physiology and morphology of the root nodules of *Podocarpus spinulosa* and *P. elata*. *Proc. Linn. Soc. N.S.W.* **48**, 82.

McLuckie, J. (1923*b*). Studies in symbiosis. IV. The root-nodules of *Casuarina cunninghamiana* and their physiological significance. *Proc. Linn. Soc. N.S.W.* **48**, 194.

McLuckie, J. (1923*c*). Studies in symbiosis. V. A contribution to the physiology of *Gastrodia sesamoides* R.Br. *Proc. Linn. Soc. N.S.W.* **48**, 436.

McNair, J. B. (1941*a*). Plant forms, the law of mass action and the production of alkaloids, cyanogenetic and organic sulphur compounds. *Amer. J. Bot.* **28**, 179.

McNair, J. B. (1941*b*). Epiphytes, parasites and geophytes and the production of alkaloids, cyanogenetic and organic sulphur compounds. *Amer. J. Bot.* **28**, 733.

McNair, J. B. (1943). Hydrophytes, xerophytes and halophytes and the production of alkaloids, cyanogenetic and organic sulphur compounds. *Lloydia*, **6**, 1.

McNew, G. L. & Spencer, E. L. (1939). Effect of nitrogen supply of sweet corn on the wilt bacterium. *Phytopathology*, **29**, 1051.

Macpherson, H. T. (1946). The basic amino acid content of proteins. *Biochem. J.* **40**, 470.

McRary, W. L. (1942). Studies on the biochemical synthesis of asparagine. *Arch. Biochem.* **1**, 197.

Magness, J. R., Batjer, L. P. & Regeimbal, L. O. (1939). Correlation of fruit color in apples to nitrogen content of leaves. *Proc. Amer. Soc. hort. Sci.* **37**, 39.

Mangels, C. E. (1925). Effect of climate and other factors on the protein content of North Dakota wheat. *Cereal Chem.* **2**, 288.

Manske, R. H. F. (1944). The alkaloids. *Ann. Rev. Biochem.* **13**, 533.

Manske, R. H. F. & Marion, L. (1942). The alkaloids of *Lycopodium* species. I. *Lycopodium complanatum* L. *Canad. J. Res.* B, **20**, 87.

Marion, L. (1939). The occurrence of *l*-nicotine in *Asclepias syriaca* L. *Canad. J. Res.* B, **17**, 21.

Markwood, L. N. (1940). Nornicotine as the predominating alkaloid in certain tobaccos. *Science*, **92**, 204.

Marthaler, H. (1937). Die Stickstoffernährung der Ruderalpflanzen. *Jb. wiss. Bot.* **85**, 76.

Martin, W. E. (1939). Nitrogen nutrition in relation to yield and quality of grapefruit. *Plant Physiol.* **14**, 606.

Martius, C. (1937). Über den Abbau der Citronensäure. *Hoppe-Seyl. Z.* **247**, 104.

Martius, C. (1938). Der intermediäre Stoffwechsel der Citronensäure. *Hoppe-Seyl. Z.* **257**, 29.

Maschmann, E. (1939). Über die Dipeptidasen anaerober Bakterien. *Naturwissenschaften*, **27**, 276.

Matuashvili, S. I. (1947). Effect of boron and molybdenum on morphology and physiology of *Azotobacter chroococcum*. *Mikrobiologiya*, **16**, 19 (Russian).

Mayer, A. & Schmidt, E. (1907). Die Wanderung der Alkaloide aus dem Pfropfreise in die Unterlage. *Ber. dtsch. bot. Ges.* **25**, 131.

Mazur, A. & Clarke, H. T. (1938). The amino acids of certain marine algae. *J. biol. Chem.* **123**, 729.

Mazur, A. & Clarke, H. T. (1942). Chemical components of some autotrophic organisms. *J. biol. Chem.* **143**, 39.

Medes, G. & Floyd, N. (1942). Metabolism of sulphur. Cysteic acid. *Biochem. J.* **36**, 836.

Menzorov, I. G. (1939). Nature of plasteins. *Biokhim.* **4**, 648 (Russian).

Meyerhof, O. (1924). *Chemical Dynamics of Life Phenomena.* Philadelphia.

Miehe, H. (1916). Über die Knospensymbiose bei *Ardisia crispa*. *Ber. dtsch. Bot. Ges.* **34**, 576.

Mikhailov, V. (1886). Transformation of peptone into albumin. *J. Russ. Phys.-Chem. Soc.* **18**, 391 (quoted from Menzorov, 1939).

Mikhlin, D. M. (1938). Hydroxylamine formed in plants in the course of nitrate and nitrite assimilation. *C.R. Acad. Sci. U.R.S.S.* **20**, 149.

Moldtmann, H. G. (1940). Untersuchungen über den Ascorbinsäuregehalt der Pflanzen in seiner Abhängigkeit von inneren und äusseren Faktoren. *Planta*, **30**, 297.

Mothes, K. (1938). Stickstoffbilanz und Stickstoffverlust. *Planta*, **28**, 599.

Mothes, K. (1940). Zur Biosynthese der Säureamide Asparagin und Glutamin. *Planta*, **30**, 726.

Mothes, K. & Hieke, K. (1943). Die Tabakwurzel als Bildungsstätte des Nikotins. *Naturwissenschaften*, **31**, 17.

Mothes, K. & Kretschmer, D. (1946). The alkaloid synthesis in isolated lupin roots. *Naturwissenschaften*, **33**, 26 (quoted from *Chem. Abstr.* **41**, 6928).

Moulder, J. W. & Evans, E. A. (1946). The biochemistry of the malaria parasite. VI. Studies on the nitrogen metabolism of the malaria parasite. *J. biol. Chem.* **164**, 145.

Mowry, H. (1933). Symbiotic nitrogen fixation in the genus *Casuarina*. *Soil Sci.* **36**, 409.

Moyer, L. S. & Fishman, M. M. (1943). The chlorophyll-protein complex. II. Species relationships in certain legumes as shown by electric motility curves. *Bot. Gaz.* **104**, 449.

Müller, E. & Armbrust, K. (1940). Über einige stickstoffhaltige Bestandteile der Sojabohne. *Hoppe-Seyl. Z.* **263**, 41.

Nagasima, H. (1938). On the developmental change of quantities of chlorophyll and carotenoid in the leaves of rice-plant, barley and wheat. *Jap. J. Bot.* **9**, 277.

Nasharty, A. H. (1944). Accumulation of nitrogen from different sources by peach trees. *Proc. Amer. Soc. hort. Sci.* **45**, 5.

Naumov, V. M. (1938). Effect of storage conditions on solanine content of potatoes. *Voprosy Pitaniya* (*Problems of Nutrition*), **7**, 208 (Russian).

Neish, A. C. & Hibbert, H. (1944). Studies on plant tumors. Part III. Nitrogen metabolism of normal and tumor tissues of the beet root. *Arch. Biochem.* **3**, 159.

Nelson, E. K. & Hasselbring, H. (1931). Some organic acids of wheat plants. *J. Amer. chem. Soc.* **53**, 1040.

Neuberger, A. & Sanger, F. (1942). The nitrogen of the potato. *Biochem. J.* **36**, 662.

NEURATH, H., GREENSTEIN, J. P., PUTNAM, F. W. & ERICKSON, J. O. (1944). The chemistry of protein denaturation. *Chem. Rev.* **34**, 157.

NEZGOVOROV, L. (1941). Zur Frage über das Vorhandensein von gebundener Amylase in den Chloroplasten. *C.R. Acad. Sci. U.R.S.S.* **30**, 260.

NIGHTINGALE, G. T. (1937*a*). The nitrogen nutrition of green plants. *Bot. Rev.* **3**, 85.

NIGHTINGALE, G. T. (1937*b*). Potassium and calcium in relation to nitrogen metabolism. *Bot. Gaz.* **98**, 725.

NIGHTINGALE, G. T. (1942*a*). Nitrate and carbohydrate reserves in relation to nitrogen nutrition of pineapple. *Bot. Gaz.* **103**, 409.

NIGHTINGALE, G. T. (1942*b*). Potassium and phosphate nutrition of pineapple in relation to nitrate and carbohydrate reserves. *Bot. Gaz.* **104**, 191.

NIGHTINGALE, G. T., ADDOMS, R. M. & BLAKE, M. A. (1930). Development and ripening of peaches as correlated with physical characteristics, chemical composition, and histological structure of the fruit flesh: III. Macrochemistry. *Bull. N.J. agric. Exp. Sta.* no. 494.

NIGHTINGALE, G. T., SCHERMERHORN, J. G. & ROBBINS, W. R. (1930). Some effects of potassium deficiency on the histological structure and nitrogenous and carbohydrate constituents of plants. *Bull. N.J. agric. Exp. Sta.* no. 499.

NOACK, K. & PIRSON, A. (1939). Die Wirkung von Eisen und Mangan auf die Stickstoffassimilation von Chlorella. *Ber. dtsch. bot. Ges.* **57**, 442.

NOGTEV, V. P. (1939). Origin and functions of nodules on the roots of the meadow foxtail (*Alopecurus pratensis* L.). *C.R. Acad. Sci. U.R.S.S.* **25**, 159.

NOGUTI, Y., OKA, H. & ÔTUKA, T. (1940). Studies on the polyploidy in *Nicotiana* induced by the treatment with colchicine. II. Growth rate and chemical analysis of diploid and its autotetraploid in *Nicotiana rustica* and *N. Tabacum. Jap. J. Bot.* **10**, 343.

NOGUTI, Y., OKUMA, K. & OKA, H. (1939). Studies on the polyploidy in *Nicotiana* induced by the treatment with colchicine. I. General observations on the autotetraploid of *Nicotiana rustica* and *N. Tabacum. Jap. J. Bot.* **10**, 309.

NOVIKOV, V. A. (1937). Derangement of metabolism in the leaves of lucerne when infected with the rust *Uromyces striatus* Schröt. *C.R. Acad. Sci. U.R.S.S.* **15**, 53.

NOWOTNÓWNA, A. (1937). An investigation of nitrogen uptake in mixed crops not receiving nitrogenous manure. *J. agric. Sci.* **27**, 503.

O'BRIEN, E. J. (1946). Victorian F.A.Q. wheat. Analysis of sample. *J. Dep. Agric. Vic.* **44**, 153.

OCHOA, S. (1944). α-ketoglutaric dehydrogenase of animal tissues. *J. biol. Chem.* **155**, 87.

OCHOA, S. (1945). Isocitric dehydrogenase and carbon dioxide fixation. *J. biol. Chem.* **159**, 243.

OCHOA, S., MEHLER, A. & KORNBERG, A. (1947). Reversible oxidative decarboxylation of malic acid. *J. biol. Chem.* **167**, 872.

OCHOA, S. & WEISZ-TABORI, E. (1945). Oxalosuccinic carboxylase. *J. biol. Chem.* **159**, 245.

OGSTON, A. G. (1943). On the theory of the periodic structure of proteins. *Trans. Faraday Soc.* **39**, 151.

O'KANE, D. E. & GUNSALUS, I. C. (1947). Aspartic-alanine transaminase, an artifact. *J. biol. Chem.* **170**, 433.

ONSLOW, M. W. (1931). Chemical effect of a mendelian factor for flower colour. *Nature, Lond.*, **128**, 373.

ONSLOW, M. W. (1932). Genetical and biochemical aspects of anthocyan pigments. *Nature, Lond.*, **129**, 601.

OPARIN, A. I. (1938). *The Origin of Life*. New York.

OPARIN, A. & GELMAN, N. (1946). Formation of purin bases in germinating wheat seeds. *C.R. Acad. Sci. U.R.S.S.* **54**, 43.

ORCUTT, F. S. (1937). Nitrogen metabolism of soy beans in relation to the symbiotic nitrogen fixation process. *Soil Sci.* **44**, 203.

OREKHOV, A. & KONOVALOVA, R. (1934). Über die Alkaloide von *Convolvulus pseudo-cantabricus* (II. Mitteil.). *Ber. dtsch. chem. Ges.* **67**, 1153.

OREKHOV, A. & KONOVALOVA, R. (1935). Über die Alkaloide von *Convolvulus pseudo-cantabricus*. III. Mitteil. Konstitution des Convolvins und Isolierung von zwei neuen Basen. *Ber. dtsch. chem. Ges.* **68**, 814.

OREKHOV, A. & MENSHIKOV, G. (1931). Über die Alkaloide von *Anabasis aphylla* L. (I. Mitteil.). *Ber. dtsch. chem. Ges.* **64**, 266.

OREKHOV, A. & PROSKURNINA, N. (1934). Über die Alkaloide von *Salsola richteri*. II. Mitteil. Die Konstitution des Salsolins. *Ber. dtsch. chem. Ges.* **67**, 878.

ÖRSTRÖM, Å. (1941). Über die chemischen Vorgänge, insbesondere den Ammoniakstoffwechsel bei der Entwicklungserregung des Seeigeleies. *Hoppe-Seyl. Z.* **271**, 1.

ÖRSTRÖM, Å., ÖRSTRÖM, M. M. & KREBS, H. A. (1939). The formation of hypoxanthine in pigeon liver. *Biochem. J.* **33**, 990.

ÖRSTRÖM, Å., ÖRSTRÖM, M., KREBS, H. A. & EGGLESTON, L. V. (1939). The synthesis of glutamine in pigeon liver. *Biochem. J.* **33**, 995.

Oudman, J. (1936). Über Aufnahme und Transport N-haltiger Verbindungen durch die Blätter von *Drosera capensis* L. *Rec. Trav. bot. néerl.* **33**, 351.

Ovcharov, K. E. (1937). The production of thiourea by fungi. *C.R. Acad. Sci. U.R.S.S.* **16**, 461.

Pal, B. P. & Nath, B. V. (1944). The accumulation and movement of nicotine in reciprocal grafts between tobacco and tomato plants. *Proc. Indian Acad. Sci.* B, **20**, 79.

Paull, A. E. & Anderson, J. A. (1942). The effects of amount and distribution of rainfall on the protein content of western Canadian wheat. *Canad. J. Res.* C, **20**, 212.

Peacock, S. M., Layerle, D. B. & Dawson, R. F. (1944). Alkaloid accumulation in reciprocal grafts of *Datura Stramonium* with tobacco and tomato. *Amer. J. Bot.* **31**, 463.

Pearsall, W. H. & Billimoria, M. C. (1937). Losses of nitrogen from green plants. *Biochem. J.* **31**, 1743.

Pearsall, W. H. & Billimoria, M. C. (1938). Effects of age and season upon protein synthesis in detached leaves. *Ann. Bot., Lond.*, N.S. **2**, 317.

Pearsall, W. H. & Billimoria, M. C. (1939). The influence of light upon nitrogen metabolism in detached leaves. *Ann. Bot., Lond.*, N.S. **3**, 601.

Pease, H. T. (1897). Poisoning of cattle by *Andropogon Sorghum*. *J. comp. Med.* **18**, 679 (quoted from Léeman (1935)).

Pennington, W. (1942). Experiments on the utilization of nitrogen in fresh water. *J. Ecol.* **30**, 326.

Pereira, A. (1944). Proteínas do trigo. I. Contribuiçao para o estudo da variaçao da proteína e gluten segundo a variedade e a regiao. *Agron. Lusitana*, **6**, 367.

Petrie, A. H. K. (1943). Protein synthesis in plants. *Biol. Rev.* **18**, 105.

Petrie, A. H. K. & Williams, R. F. (1938). Physiological ontogeny in plants and its relation to nutrition. 5. The effect of nitrogen and phosphorus supplies on the respiration rate of the leaves. *Aust. J. exp. Biol. med. Sci.* **16**, 347.

Petrie, A. H. K. & Wood, J. G. (1938*a*). Studies on the nitrogen metabolism of plants. I. The relation between the content of proteins, amino acids, and water in the leaves. *Ann. Bot., Lond.*, N.S. **2**, 33.

Petrie, A. H. K. & Wood, J. G. (1938*b*). Studies on the nitrogen metabolism of plants. III. On the effect of water content on the relationship between proteins and amino-acids. *Ann. Bot., Lond.*, N.S. **2**, 887.

Petrie, T. M. (1917). The chemical investigation of some poisonous plants in the N.O. Solanaceae. Part IV. The chemistry of the Duboisias. *Proc. Linn. Soc. N.S.W.* **42**, 118.

Petrov, G. G. (1917). The assimilation of nitrogen by seed-plants in the light and in darkness (quoted from Kostychev, S. P., *Chemical Plant Physiology*, p. 102. Philadelphia, 1931).

Phillips, J. (1932). Root nodules of *Podocarpus*. *Ecology*, **13**, 189.

Phillis, E. & Mason, T. G. (1942). Studies on the partition of the mineral elements in the cotton plant. III. Mainly concerning nitrogen. *Ann. Bot., Lond.*, N.S. **6**, 468.

Piatnitski, M. P. (1940*a*). Effect of some organic acid salts on storage of citric acid by leaves of tobacco plants. *C.R. Acad. Sci. U.R.S.S.* **29**, 55.

Piatnitski, M. P. (1940*b*). Effect of mineral salts on storage of citric acid by leaves of *Nicotiana rustica* and *Nicotiana glauca*. *C.R. Acad. Sci. U.R.S.S.* **29**, 59.

Pietz, J. (1938). Beitrag zur Physiologie des Wurzelknöllchenbakteriums. *Zbl. Bakt.* 2te Abteil. **99**, 1.

Pinckney, R. M. (1924). Effect of nitrate applications upon the hydrocyanic-acid content of sorghum. *J. agric. Res.* **27**, 717.

Piper, C. S. (1940). Molybdenum as an essential element for plant growth. *J. Aust. Inst. agric. Sci.* **6**, 162.

Pirschle, K. (1929). Zur Assimilation des Harnstoffs durch die höhere Pflanze. *Biochem. Z.* **212**, 466.

Plentl, A. A. & Schoenheimer, R. (1944). Studies in the metabolism of purines and pyrimidines by means of isotopic nitrogen. *J. biol. Chem.* **153**, 203.

Postma, W. P. (1939). Einige Bemerkungen über den Einfluss der Nitratreduktion auf die Atmung der Wurzeln. *Proc. K. Akad. Amst.* **42**, 181.

Prasad, S. (1946). The influence of fertilizers on the growth and alkaloidal content of *Hyoscyamus niger* Linn. *J. Amer. pharm. Ass.* **35**, 121.

Pratesi, P. & Ciferri, R. (1946). Studi sul biochimismo del tabacco. I. Influenza di alcuni fattori del bios sullo sviluppo dei germinelli. *Boll. Soc. ital. biol. Sper.* **21**, 182.

Pratesi, P., Ciferri, R. & Cambieri, F. (1946). Studi sul biochimismo del tabacco. IV. Ancora sull' acido nicotinico e derivati in relazione alla genesi degli alcaloidi. *Boll. Soc. ital. biol. Sper.* **21**, 250.

Pryanishnikov, D. N. (1928). Über die Ausscheidung von Ammoniak durch die Pflanzenwurzeln bei Säurevergiftung. *Biochem. Z.* **193**, 211.

Przylecki, S. J. (1939). Protein symplexes. *Proc. roy. Soc.* A, **170**, 65.

Pucher, G. W., Leavenworth, C. S., Ginter, W. D. & Vickery, H. B. (1947*a*). Studies in the metabolism of crassulacean plants: changes in the composition of *Bryophyllum calycinum* during growth. *Plant Physiol.* **22**, 1.

Pucher, G. W., Leavenworth, C. S., Ginter, W. D. & Vickery, H. B. (1947*b*). Studies in the metabolism of crassulacean plants: the effect upon the composition of *Bryophyllum calycinum* of the form in which nitrogen is supplied. *Plant Physiol.* **22**, 205.

Pucher, G. W. & Vickery, H. B. (1941). Succinic acid as a metabolite in plant tissues. *Plant Physiol.* **16**, 771.

PUCHER, G. W. & VICKERY, H. B. (1942). On the identity of the so-called crassulacean malic acid with isocitric acid. *J. biol. Chem.* **145**, 525.

PUCHER, G. W., WAKEMAN, A. J. & VICKERY, H. B. (1938). The organic acids of rhubarb (*Rheum hybridum*). III. The behaviour of the organic acids during culture of excised leaves. *J. biol. Chem.* **126**, 43.

PUCHER, G. W., WAKEMAN, A. J. & VICKERY, H. B. (1939). Organic acid metabolism of the buckwheat plant. *Plant Physiol.* **14**, 333.

RAISTRICK, H. & CLARK, A. B. (1919). On the mechanism of oxalic acid formation by *Aspergillus niger*. *Biochem. J.* **13**, 329.

RALEIGH, G. J. (1946). Glutamine from rye grass. *Science*, **103**, 206.

RAO, K. A. (1923). A preliminary account of symbiotic nitrogen fixation in non-leguminous plants with special reference to *Chomelia asiatica*. *Agric. J. India*, **18**, 132.

RAOUL, Y. (1937*a*). Nouvelle synthèse de l'hordénine. *C.R. Acad. Sci., Paris*, **204**, 74.

RAOUL, Y. (1937*b*). Évolution de l'hordénine dans l'orge et relations éventuelles de cet alcaloïde avec la tyrosine. *C.R. Acad. Sci., Paris*, **205**, 450.

RATNER, S. (1947). The enzymatic mechanism of arginine formation from citrulline. *J. biol. Chem.* **170**, 761.

RAUP, H. M. (1941). Botanical problems in boreal America. I. *Bot. Rev.* **7**, 147.

RAUTANEN, N. (1946). Transamination in green plants. *J. biol. Chem.* **163**, 687.

RAVEL, J. M. & SHIVE, W. (1946). Biochemical transformations as determined by competitive analogue-metabolite growth inhibitions. IV. Prevention of pantothenic acid synthesis by cysteic acid. *J. biol. Chem.* **166**, 407.

RAYNER, M. C. (1922). Nitrogen fixation in Ericaceae. *Bot. Gaz.* **73**, 227.

REES, M. W. (1946). The estimation of threonine and serine in proteins. *Biochem. J.* **40**, 632.

RICHARDS, F. J. (1938). Physiological studies in plant nutrition. VIII. The relation of respiration rate to the carbohydrate and nitrogen metabolism of the barley leaf as determined by phosphorus and potassium supply. *Ann. Bot., Lond.*, N.S. **2**, 491.

RICHARDSON, A. E. V., TRUMBLE, H. C. & SHAPTER, R. E. (1932). The influence of growth stage and frequency of cutting on the yield and composition of a perennial grass—*Phalaris tuberosa*. *Bull. Coun. sci. industr. Res. Aust.* no. 66.

RICHARDSON, C. & CRAMPTON, C. A. (1886). Vorläufige Mittheilung über die Zusammensetzung des Weizenkeimes und über die Anwesenheit von einen neuen Zuckerart und von Allantoin. *Ber. dtsch. chem. Ges.* **19**, 1180.

RIMINGTON, C. & QUIN, J. I. (1933). Studies in the photosensitisation of animals in South Africa. II. The presence of a lethal factor in certain members of the plant genus *Tribulus*. *Onderstepoort J. vet. Sci.* **1**, 469.

RISHKOV, V. L. & SMIRNOVA, V. A. (1939). Accumulation of virus of tobacco mosaic in plants when nitrogen is withheld from them. *C.R. Acad. Sci. U.R.S.S.* **23**, 95.

RITTENBERG, D. & BLOCH, K. (1945). Some biological reactions of acetic acid. *J. biol. Chem.* **157**, 749.

RITTENBERG, D., SCHOENHEIMER, R. & KESTON, A. S. (1939). Studies in protein metabolism. IX. The utilisation of ammonium by normal rats on a stock diet. *J. biol. Chem.* **128**, 603.

RITTENBERG, D. & WAELSCH, H. (1940). The source of carbon for urea formation. *J. biol. Chem.* **136**, 799.

ROBBINS, W. J. & MA, R. (1945). Growth factors for *Trichophyton mentagrophytes*. *Amer. J. Bot.* **32**, 509.

ROBERG, M. (1934). Weitere Untersuchungen über die *N*-Ernährung der Erle. *Ber. dtsch. bot. Ges.* **52**, 54.

ROBERG, M. (1936). Beiträge zur Biologie von *Azotobacter*. III. Zur Frage eines ausserhalb der Zelle den Stickstoff bindenden Enzyms. *Jb. wiss. Bot.* **83**, 567.

ROBINSON, R. (1936). Synthesis in biochemistry. *J. chem. Soc.* p. 1079.

ROINE, P. (1946*a*). On the synthesis of nitrogenous compounds by yeast. II. The soluble compounds formed during the uptake of ammonium nitrogen by low-nitrogen yeast. *Suomen Kemistilehti*, B, **19**, 73.

ROINE, P. (1946*b*). On the rôle of glutamine in the protein synthesis by yeast. *Suomen Kemistilehti*, B, **19**, 113.

ROLOFF, M., RATNER, S. & SCHOENHEIMER, R. (1940). The biological conversion of ornithine into proline and glutamic acid. *J. biol. Chem.* **136**, 561.

ROMASHEV, P. I. (1939). Utilisation of the nitrogen of leguminous herbs in meadow-farming. *Pochvovedenie* (*Pedology*), no. 4, p. 99 (Russian).

ROTMISTROV, M. N. (1941). Die Assimilation des Luftstickstoffs durch anaerobe Zellulosebakterien. *C.R. Acad. Sci. U.R.S.S.* **32**, 230.

ROWSON, J. M. (1944*a*). Increased alkaloidal contents of induced polyploids of *Datura*. *Nature, Lond.*, **154**, 81.

ROWSON, J. M. (1944*b*). The assay of *Belladonna* and of some other solanaceous drugs—determination of hyoscyamine, atropine and hyoscine. *Quart. J. Pharm.* **17**, 234.

RUBEN, S., HASSID, W. Z. & KAMEN, M. D. (1940*a*). Radioactive nitrogen in the study of nitrogen fixation by non-leguminous plants. *Science*, **91**, 578.

RUBEN, S., HASSID, W. Z. & KAMEN, M. D. (1940*b*). Photosynthesis with radioactive carbon. II. Chemical properties of the intermediates. *J. Amer. chem. Soc.* **62**, 3443.

Rubin, B. A. (1945). *Biochemical Basis of Vegetable Storage*. Moscow-Leningrad (Russian).

Rubin, B. A., Artsikhovskaya, E. V. & Proskurnikova, T. A. (1946). Vitamin C and the oxidative activity of plant tissues. IV. Role of ascorbic acid in rose hips. *Biokhim.* **11**, 349 (Russian).

Sabalitschka, T. & Jungermann, C. (1926). Der absolute und prozentuale Alkaloidgehalt der einzelnen Teile der Keimlinge und der jungen Pflanze von *Strychnos nux-vomica* L. während der Keimung. *Biochem. Z.* **167**, 479.

Sabet, Y. S. (1946). Bacterial root nodules in the Zygophyllaceae. *Nature, Lond.*, **157**, 656.

Said, H. & el Shishiny, E. D. H. (1944). The effect of disc thickness on the respiration and the various nitrogen fractions of cut discs of radish roots immersed in water and in sugar solutions. *Plant Physiol.* **19**, 660.

Sanford, F. (1914). An experiment on killing tree scale by poisoning the sap of the tree. *Science*, **40**, 519.

Schales, O., Mims, V. & Schales, S. S. (1946). Glutamic acid decarboxylase of higher plants. I. Distribution; preparation of clear solutions; nature of prosthetic group. *Arch. Biochem.* **10**, 455.

Schales, O. & Schales, S. S. (1946). Glutamic acid decarboxylase of higher plants. II. pH-activity curve, reaction kinetics, inhibition by hydroxylamine. *Arch. Biochem.* **11**, 155.

Schanderl, H. (1943). Untersuchungen über den Stickstoffhaushalt von Nichtleguminosen und Leguminosen. *Planta*, **33**, 424.

Scharnagel, T. & Aufhammer, G. (1942). Zur Beurteilung der Klebermenge des Weizens, insbesondere die Abhängigkeit von äusseren und inneren Faktoren. *Forschungsdienst*, **14**, 165.

Schlenk, F. & Fisher, A. (1945). Note on the purification and properties of glutamic-aspartic transaminase. *Arch. Biochem.* **8**, 337.

Schlenk, F. & Fisher, A. (1947). Studies on glutamic-aspartic acid transaminase. *Arch. Biochem.* **12**, 69.

Schlenk, F. & Snell, E. E. (1945). Vitamin B_6 and transamination. *J. biol. Chem.* **157**, 426.

Schneider, A. (1942). Über das Auftreten der Milchsäure in höheren Pflanzen, insbesondere während der Keimung. *Planta*, **32**, 234.

Schneider, K. (1935). Beeinflussung von *N*-Stoffwechsel und Stengelanatomie durch Ernährung (Weitere Untersuchungen an *Pelargonium*). *Z. Bot.* **29**, 545.

Schoenheimer, R., Ratner, S., Rittenberg, D. & Heidelberger, M. (1942). The interaction of the blood proteins of the rat with dietary nitrogen. *J. biol. Chem.* **144**, 541.

Schöpf, C. (1937). Die Synthese von Naturstoffen, insbesondere von Alkaloiden, unter physiologischen Bedingungen und ihre Bedeutung für die Frage der Entstehung einiger pflanzlicher Naturstoffe in der Zelle. *Angew. Chem.* **50**, 779, 797.

Schopfer, W. H. (1943). *Plants and Vitamins*. Waltham, Mass.

Schroeder, W. T. & Walker, J. C. (1942). Influence of controlled environment and nutrition on the resistance of garden peas to *Fusarium* wilt. *J. agric. Res.* **65**, 221.

Schwabe, G. (1932). Über die Wirkung der Aminosäuren auf den Sauerstoffverbrauch submerser Gewachse. *Protoplasma*, **16**, 397.

Shapter, R. E. (1939). An experiment to detect the possible excretion of nitrogen by leguminous plants. *J. Coun. sci. industr. Res. Aust.* **12**, 23.

Shcherbakov, A. P. (1945). Changes of respiration in plants deprived of potassium. *Biokhim.* **10**, 439 (Russian).

Shear, G. M. & Wingard, S. A. (1944). Some ways by which nutrition may affect severity of disease in plants. *Phytopathology*, **34**, 603.

Shemin, D. (1946). The biological conversion of *l*-serine to glycine. *J. biol. Chem.* **162**, 297.

Shemin, D. & Foster, G. L. (1946). The isotope dilution method of amino acid analysis. *Ann. N.Y. Acad. Sci.* **47**, 119.

Shemin, D. & Rittenberg, D. (1944). Some interrelationships in general nitrogen metabolism. *J. biol. Chem.* **153**, 401.

Shive, W. & Macow, J. (1946). Biological transformations as determined by competitive analogue-metabolite growth inhibitions. I. Some transformations involving aspartic acid. *J. biol. Chem.* **162**, 451.

Shive, W. & Rogers, L. L. (1947). Involvement of biotin in the biosynthesis of oxalacetic and α-keto-glutaric acids. *J. biol. Chem.* **169**, 453.

Shmuk, A. A. (1937). Chemical composition of interspecific hybrids of the genus *Nicotiana*. *Bull. Acad. Sci. U.R.S.S.*, Sér. Biol. p. 1693 (Russian).

Shmuk, A. A. (1940). Changes in chemical composition in grafted plants. *Dokl. Vsesoyuz. Akad. S.-Kh. Nauk imeni Lenina*, no. 11, p. 9 (Russian).

Shmuk, A. A. (1945). Biochemical changes in grafted plants, and grafting of plants in the solution of physiological and biochemical problems. *Dokl. Vsesoyuz. Akad. S.-Kh. Nauk imeni Lenina*, p. 3 (Russian).

Shmuk, A. A. & Borozdina, A. (1941). Alkaloids of different species of the genus *Nicotiana*. *C.R. Acad. Sci. U.R.S.S.* **32**, 62 (Russian).

Shmuk, A. A., Kostov, D. & Borozdina, A. (1939). Alteration of the alkaloid composition due to the influence of stock upon scion in *Nicotiana*. *C.R. Acad. Sci. U.R.S.S.* **25**, 477.

SHMUK, A. A., PISAREV, V. & VINOGRADOVA, N. (1944). Change in properties of wheat grown on rye endosperm. *Dokl. Vsesoyuz. Akad. S.-Kh. Nauk imeni Lenina*, no. 7, p. 9 (Russian).

SHMUK, A. A., SMIRNOV, A. & ILYIN, G. (1941). Formation of nicotine in plants grafted on tobacco. *C.R. Acad. Sci. U.R.S.S.* **32**, 365.

SIDERIS, C. P. (1947). Chlorophyll and protein interrelationships in *Ananas comosus* (L.). Merr. *Plant Physiol.* **22**, 160.

SIDERIS, C. P. & KRAUSS, B. H. (1937). The growth of pineapple plants in complete water cultures with either ammonia or nitrate salts. *Growth*, **1**, 204.

SIDERIS, C. P., KRAUSS, B. H. & YOUNG, H. Y. (1937). Assimilation of ammonium and nitrate nitrogen from solution cultures by roots of *Pandanus veitchii* Hort., and distribution of the various nitrogen fractions and sugars in the stele and cortex. *Plant Physiol.* **12**, 899.

SIDERIS, C. P., KRAUSS, B. H. & YOUNG, H. Y. (1938*a*). Assimilation of ammonium and nitrate by pineapple plants grown in nutrient solutions and its effects on nitrogenous and carbohydrate constituents. *Plant Physiol.* **13**, 489.

SIDERIS, C. P., KRAUSS, B. H. & YOUNG, H. Y. (1938*b*). The distribution of uncombined hexosamine in pineapple plants supplied either with ammonium sulfate or calcium nitrate salts. *J. biol. Chem.* **126**, 233.

SIDERIS, C. P., KRAUSS, B. H. & YOUNG, H. Y. (1939). Distribution of different nitrogen fractions, sugars and other substances in various sections of the pineapple plant grown in soil cultures and receiving either ammonium or nitrate salts. *Plant Physiol.* **14**, 227.

SIDERIS, C. P. & YOUNG, H. Y. (1945). Effects of different amounts of potassium on growth and ash constituents of *Ananas comosus* (L.) Merr. *Plant Physiol.* **20**, 609.

SIDERIS, C. P. & YOUNG, H. Y. (1946). Effect of nitrogen on growth and ash constituents of *Ananas comosus* (L.) Merr. *Plant Physiol.* **21**, 247.

SIDERIS, C. P. & YOUNG, H. Y. (1947). Effects of nitrogen on chlorophyll, acidity, ascorbic acid, and carbohydrate fractions of *Ananas comosus* (L.) Merr. *Plant Physiol.* **22**, 97.

SIDERIS, C. P., YOUNG, H. Y. & CHUN, H. H. Q. (1947). Effects of nitrogen on the nitrogenous fractions of *Ananas comosus* (L.) Merr. *Plant Physiol.* **22**, 127.

SIMMONDS, S., COHN, M., CHANDLER, J. P. & DU VIGNEAUD, V. (1943). The utilisation of the methyl groups of choline in the biological synthesis of methionine. *J. biol. Chem.* **149**, 519.

SIMMONDS, S. & DU VIGNEAUD, V. (1942). Transmethylation as a metabolic process in man. *J. biol. Chem.* **146**, 685.

SIMMONDS, S., TATUM, E. L. & FRUTON, J. S. (1947). The utilisation of phenylalanine and tyrosine derivatives by mutant strains of *Escherichia coli*. *J. biol. Chem.* **169**, 91.

SINCLAIR, W. B. & LINDGREN, D. L. (1943). Ridges and sectors induced in the rind of citrus fruits by fumigation with hydrocyanic acid. *Plant Physiol.* **18**, 99.

SINCLAIR, W. B. & LINDGREN, D. L. (1946). Ridges and sectors induced in olive fruits by fumigation with hydrocyanic acid. *Plant Physiol.* **21**, 369.

SKOK, J. (1941). Effect of the form of the available nitrogen on the calcium deficiency symptoms in the bean plant. *Plant Physiol.* **16**, 145.

SMITH, A. M. & ROBB, W. (1943). The carotene and protein in oats and barley at different stages of growth. *J. agric. Sci.* **33**, 119.

SMITH, A. M. & WANG, T. (1941). The carotene content of certain species of grassland herbage. *J. agric. Sci.* **31**, 370.

SMITH, E. L., GREEN, R. D. & BARTNER, E. (1946). Amino-acid composition of seed globulins. *J. biol. Chem.* **164**, 159.

SMITH, H. H. & SMITH, C. R. (1942). Alkaloids in certain species and interspecific hybrids of *Nicotiana*. *J. agric. Res.* **65**, 347.

SMYTHE, C. V. (1944). Some enzyme reactions of sulfur compounds and their possible interrelationship. *Ann. N.Y. Acad. Sci.* **45**, 425.

SNELL, E. E. (1944). The vitamin activities of 'pyridoxal' and 'pyridoxamine'. *J. biol. Chem.* **154**, 313.

SNELL, E. E. (1945*a*). The vitamin B_6 group. IV. The occurrence of pyridoxamine and pyridoxal in natural products. *J. biol. Chem.* **157**, 491.

SNELL, E. E. (1945*b*). The vitamin B_6 group. V. The reversible interconversion of pyridoxal and pyridoxamine by transamination reactions. *J. Amer. chem. Soc.* **67**, 194.

SNELL, E. E. (1946). Microbiological methods in amino acid analysis. *Ann. N.Y. Acad. Sci.* **47**, 161.

SOLTYS, A. & WALLENFELS, K. (1936). Solanin und Solanidin. *Ber. dtsch. chem. Ges.* **69**, 811.

SPÄTH, E. (1929). Über das Carnegin. *Ber. dtsch. chem. Ges.* **62**, 1021.

SPÄTH, E., HICKS, C. S. & ZAJIC, E. (1935). *d*-Nor-nicotin, ein Alkaloid von *Duboisia hopwoodii* F. v. Muell. *Ber. dtsch. chem. Ges.* **68**, 1388.

SPECK, J. F. (1947). The enzymatic synthesis of glutamine. *J. biol. Chem.* **168**, 403.

SPENCER, E. L. (1941). Inhibition of increase and activity of tobacco-mosaic virus under nitrogen-deficient conditions. *Plant Physiol.* **16**, 227.

Spiegelman, S. & Kamen, M. D. (1946). Genes and nucleoproteins in the synthesis of enzymes. *Science*, **104**, 581.

Spratt, E. R. (1912*a*). The morphology of the root tubercles of *Alnus* and *Elaeagnus*, and the polymorphism of the organisms causing their formation. *Ann. Bot., Lond.*, **26**, 119.

Spratt, E. R. (1912*b*). The formation and physiological significance of root nodules in the Podocarpineae. *Ann. Bot., Lond.*, **26**, 801.

Srb, A. M. & Horowitz, N. H. (1944). The ornithine cycle in *Neurospora* and its genetic control. *J. biol. Chem.* **154**, 129.

Stare, F. J. & Baumann, C. A. (1936). The effect of fumarate on respiration. *Proc. roy. Soc.* B, **121**, 338.

Steinberg, R. A. (1936). Relation of accessory growth substances to heavy metals, including molybdenum, in the nutrition of *Aspergillus niger*. *J. agric. Res.* **52**, 439.

Steinberg, R. A. (1937). Rôle of molybdenum in the utilization of ammonium and nitrate nitrogen by *Aspergillus niger*. *J. agric. Res.* **55**, 891.

Steinberg, R. A. (1939). Effects of nitrogen compounds and trace elements on growth of *Aspergillus niger*. *J. agric. Res.* **59**, 731.

Steinberg, R. A. (1941). Use of *Lemna* for nutrition studies on green plants. *J. agric. Res.* **62**, 423.

Steinhardt, J. & Fugitt, C. H. (1942). Catalysed hydrolysis of amide and peptide bonds in proteins. *Bur. Stand. J. Res. Wash.* **29**, 315.

Stetten, D. (1942). The fate of dietary serine in the body of the rat. *J. biol Chem.* **144**, 501.

Steward, F. C., Berry, W. E., Preston, C. & Ramamurti, T. K. (1943). The absorption and accumulation of solutes by living plant cells. X. *Ann. Bot.* N.S. **7**, 221.

Steward, F. C. & Preston, C. (1940). Metabolic processes of potato discs under conditions conducive to salt accumulation. *Plant Physiol.* **15**, 23.

Steward, F. C. & Preston, C. (1941*a*). The effect of salt concentration upon the metabolism of potato discs and the contrasted effect of potassium and calcium salts which have a common ion. *Plant Physiol.* **16**, 85.

Steward, F. C. & Preston, C. (1941*b*). Effects of pH and the components of bicarbonate and phosphate buffered solutions on the metabolism of potato discs and their ability to absorb ions. *Plant Physiol.* **16**, 481.

Steward, F. C., Stout, P. R. & Preston, C. (1940). The balance sheet of metabolites for potato discs showing the effect of salts and dissolved oxygen on metabolism at 23° C. *Plant Physiol.* **15**, 409.

Steward, F. C. & Street, H. E. (1946). The soluble nitrogen fractions of potato tubers; the amides. *Plant Physiol.* **21**, 155.

Stokes, J. L., Larsen, A. & Gunness, M. (1946). Biotin and the synthesis of aspartic acid by microorganisms. *J. biol. Chem.* **167**, 613.

Strasburger, E. (1885). Über Verwachsungen und deren Folgen. *Ber. dtsch. bot. Ges.* **3**, 39.

Strebeyko, P. (1939). Über den Einfluss des Phosphors auf den *N*-Stoffwechsel des Hafers. Vorläufige Mitteilung. *Planta*, **29**, 477.

Street, H. E., Kenyon, A. E. & Watson, G. M. (1946*a*). The assimilation of ammonium and nitrate nitrogen by detached potato sprouts. *Ann. appl. Biol.* **33**, 369.

Street, H. E., Kenyon, A. E. & Watson, G. M. (1946*b*). The estimation of free choline in plants. *Biochem. J.* **40**, 869.

Strogonov, B. P. & Ostapenko, L. A. (1946). On the intoxication of cotton plants with ammonia when grown under conditions of increased soil salinity. *C.R. Acad. Sci. U.R.S.S.* **54**, 365.

Strong, T. H. & Trumble, H. C. (1939). Excretion of nitrogen by leguminous plants. *Nature, Lond.*, **143**, 286.

Stuart, N. W. & Appleman, C. O. (1935). Nitrogenous metabolism in Irish potatoes during storage. *Bull. Md. agric. Exp. Sta.* no. 372.

Suneson, S. (1932). Über Nitratspeicherung bei höheren Meersalgen. *Hoppe-Seyl. Z.* **204**, 81.

Suneson, S. (1933). Weitere Angaben über die Nitratspeicherung bei den höheren Meersalgen. *Hoppe-Seyl. Z.* **214**, 105.

Suomalainen, H. (1940). On the chemical mechanism of the biological nitrogen fixation. *Suomen Kemistilehti*, B, **13**, 17.

Suzuki, U., Shimamura, T. & Odake, S. (1912). Über Oryzanin, ein Bestandteil der Reiskleie und seine physiologische Bedeutung. *Biochem. Z.* **43**, 89.

Swaby, R. J. (1945). Do legumes excrete nitrogenous compounds? *J. Aust. Inst. agric. Sci.* **11**, 23.

Szent-Györgyi, A. (1936). Über die Bedeutung der Fumarsäure für die tierische Gewebsatmung. III. Mitteilung. Einleitung, Übersicht, Methoden. *Hoppe-Seyl. Z.* **244**, 105.

Takahashi, W. N. (1941). Changes in nitrogen and virus content of detached tobacco leaves in darkness. *Phytopathology*, **31**, 1117.

Tanaka, I. (1931). Studien über die Ernährung der höheren Pflanzen mit den organischen Verbindungen. *Jap. J. Bot.* **5**, 323.

Tatum, E. L. & Bonner, D. (1944). Indole and serine in the biosynthesis and breakdown of tryptophane. *Proc. nat. Acad. Sci., Wash.*, **30**, 30.

TATUM, E. L., BONNER, D. & BEADLE, G. W. (1944). Anthranilic acid and the biosynthesis of indole and tryptophane by *Neurospora*. *Arch. Biochem.* **3**, 477.

TAYLOR, E. S. & GALE, E. F. (1945). Studies on bacterial aminoacid decarboxylases. 6. Codecarboxylase content and action of inhibitors. *Biochem. J.* **39**, 52.

TERNOVSKI, M. F., KHMURA, M. I. & ZHUKOV, N. I. (1937). Inheritance of nicotine and anabasine in interspecific hybrids with *Nicotiana glauca* Gr. *C.R. Acad. Sci. U.R.S.S.* **17**, 43.

THIERFELDER, H. & SHERWIN, C. P. (1914). Phenylacetyl-glutamin, ein Stoffwechsel-Produkt des menschlichen Körpers nach Eingabe von Phenyl-essigsäure. *Ber. dtsch. chem. Ges.* **47**, 2630.

THORNTON, H. G. (1939). Discussion of paper by L. T. LEONARD, *Trans. Third Comm. Int. Soc. Soil Sci.* B, p. 23.

THORP, F., DEEM, A. W., HARRINGTON, H. D. & TOBISKA, J. W. (1937). *Suckleya Suckleyana.* A poisonous plant. *Bull. Colo. agric. Exp. Sta.* no. 22.

TOENNIES, G. & CALLAN, T. P. (1939). Methionine studies. III. A comparison of oxidative reactions of methionine, cysteine and cystine. Determination of methionine by hydrogen peroxide oxidation. *J. biol. Chem.* **129**, 481.

TRAUTNER, E. M. (1947). Alkaloid formation in *Duboisia myoporoides* and *D. Leichhardtii*. *Aust. Chem. Inst. J. Proc.* **14**, 411.

TRAUTNER, E. M. & NEUFELD, O. E. (1946). The titrimetric determination of diacidic bases (nicotine, quinine) in mixtures with monacidic bases, and a simplified assay of nicotine. *Aust. Chem. Inst. J. Proc.* **13**, 70.

TRAUTNER, E. M. & NEUFELD, O. E. (1947). The occurrence of ursolic acid in the leaves of *Duboisia* sp. *Aust. Chem. Inst. J. Proc.* **14**, 17.

TRISTRAM, G. R. (1946). Observations upon the application of partition chromatography to the determination of the monamino-acids in proteins. *Biochem. J.* **40**, 721.

TRUMBLE, H. C. & DAVIES, J. G. (1934). The establishment, persistency, and productivity of selected pasture species on an irrigated reclaimed swamp. *Bull. Coun. sci. industr. Res. Aust.* no. 80.

TRUMBLE, H. C. & FERRES, H. M. (1946). Responses of herbage legumes to applied nutrients on some southern Australian soils and their dependence on external factors. *J. Aust. Inst. agric. Sci.* **12**, 32.

TRUMBLE, H. C. & SHAPTER, R. E. (1937). Investigations on the associated growth of herbage plants. 2. The influence of nitrogen and phosphorus treatments on the yield and chemical composition of Wimmera rye grass and subterranean clover, grown separately and in association. 3. The yield and nitrogen content of a perennial grass (*Phalaris tuberosa*) when grown in association with perennial legumes. *Bull. Coun. sci. industr. Res. Aust.* no. 105, p. 25.

TRUMBLE, H. C. & STRONG, T. H. (1937). Investigations on the associated growth of herbage plants. 1. On the nitrogen accretion of pasture grasses when grown in association with legumes. *Bull. Coun. sci. industr. Res. Aust.* no. 105, p. 11.

TUBA, J., HUNTER, G. & OSBORNE, J. A. (1946). On staining for vitamin C in tissues. *Canad. J. Res.* C, **24**, 182.

TURKOVA, N. S. (1945). Effect of ethylene on the biochemistry of sugar beet roots in connection with dormancy. *Biokhim.* **10**, 385 (Russian).

UGRYMOV, P. S. (1940). Synthesis of the alkaloid arecoline. *C.R. Acad. Sci. U.R.S.S.* **29**, 48.

UMBREIT, W. W. & BURRIS, R. H. (1938). Composition of soybean nodules and root nodule bacteria. *Soil Sci.* **45**, 111.

UMBREIT, W. W. & GUNSALUS, I. C. (1945). The function of pyridoxine derivatives: arginine and glutamic acid decarboxylases. *J. biol. Chem.* **159**, 333.

UMBREIT, W. W., WOOD, W. A. & GUNSALUS, I. C. (1946). The activity of pyridoxal phosphate in tryptophane formation by cell-free enzyme preparations. *J. biol. Chem.* **165**, 731.

USSING, H. H. (1945). Isolation of asparagine from the haemolymph of *Melolontha* larvae. *Nature, Lond.*, **155**, 481.

UTTER, M. F. & WOOD, H. G. (1945). Fixation of carbon dioxide in oxalacetate by pigeon liver. *J. biol. Chem.* **160**, 375.

VAN ITERSON, G., MEYER, K. H. & LOTMAR, W. (1936). Über den Feinbau des pflanzlichen Chitins. *Rec. Trav. chim. Pays-Bas*, **55**, 61.

VAN SLYKE, D. D., PHILLIPS, R. A., HAMILTON, P. B., ARCHIBALD, R. M., FUTCHER, P. H. & HILLER, A. (1943). Glutamine as source of urinary ammonia. *J. biol. Chem.* **150**, 481.

VENNESLAND, B., CEITHAML, J. & GOLLUB, M. C. (1947). The fixation of carbon dioxide in a plant tricarboxylic acid system. *J. biol. Chem.* **171**, 445.

VENNESLAND, B. & FELSHER, R. Z. (1946). Oxalacetic and pyruvic carboxylases in some dicotyledonous plants. *Arch. Biochem.* **11**, 279.

VICKERY, H. B. (1945). The proteins of plants. *Physiol. Rev.* **25**, 347.

VICKERY, H. B. & PUCHER, G. W. (1939). The metabolism of amides in green plants. III. The mechanism of amide synthesis. *J. biol. Chem.* **126**, 703.

VICKERY, H. B. & PUCHER, G. W. (1943). Amide metabolism in etiolated seedlings. I. Asparagine and glutamine formation in *Lupinus angustifolius*, *Vicia atropurpurea* and *Cucurbita pepo*. *J. biol. Chem.* **150**, 197.

Vickery, H. B., Pucher, G. W., Leavenworth, C. S. & Wakeman, A. J. (1935). Chemical investigations of the tobacco plant. V. Chemical changes that occur during growth. *Bull. Conn. agric. Exp. Sta.* no. 374.

Vickery, H. B., Pucher, G. W., Leavenworth, C. S. & Wakeman, A. J. (1938). The metabolism of amides in green plants. II. The amides of the rhubarb leaf. *J. biol. Chem.* **125**, 527.

Vickery, H. B., Pucher, G. W., Schoenheimer, R. & Rittenberg, D. (1939). The metabolism of nitrogen in the leaves of the buckwheat plant. *J. biol. Chem.* **129**, 791.

Vickery, H. B., Pucher, G. W., Schoenheimer, R. & Rittenberg, D. (1940). The assimilation of ammonia nitrogen by the tobacco plant: a preliminary study with isotopic nitrogen. *J. biol. Chem.* **135**, 531.

Vickery, H. B., Pucher, G. W., Wakeman, A. J. & Leavenworth, C. S. (1933). Chemical investigations of the tobacco plant. *Publ. Carneg. Instn*, no. 445.

Vickery, H. B., Pucher, G. W., Wakeman, A. J. & Leavenworth, C. S. (1937). Chemical investigations of the tobacco plant. VI. Chemical changes that occur in leaves during culture in light and in darkness. *Bull. Conn. agric. Exp. Sta.* no. 399.

Vickery, H. B., Pucher, G. W., Wakeman, A. J. & Leavenworth, C. S. (1939). Chemical investigations of the rhubarb plant. *Bull. Conn. agric. Exp. Sta.* no. 424.

Vickery, H. B., Pucher, G. W., Wakeman, A. J. & Leavenworth, C. S. (1940). Chemical investigations of the tobacco plant. VIII. The effect upon the composition of the tobacco plant of the form in which nitrogen is supplied. *Bull. Conn. agric. Exp. Sta.* no. 442.

Vickery, H. B., Pucher, G. W., Wakeman, A. J. & Leavenworth, C. S. (1946). Chemical investigations of the metabolism of plants. I. The nitrogen nutrition of *Narcissus Poeticus*. *Bull. Conn. agric. Exp. Sta.* no. 496.

Vickery, H. B., Smith, E. L., Hubbell, R. B. & Nolan, L. S. (1941). Cucurbit seed globulins. I. Amino acid composition and preliminary tests of nutritive value. *J. biol. Chem.* **140**, 613.

Viets, F. G. (1945). The rôle of amino acids and amides in the metabolism of ammonium absorbed by *Zea mays* L. *Science*, **102**, 587.

Viets, F. G., Moxon, A. L. & Whitehead, E. I. (1946). Nitrogen metabolism of corn (*Zea mays*) as influenced by ammonium nutrition. *Plant Physiol.* **21**, 271.

Vinogradova, K. G. (1943). Presence of molybdenum in Leguminosae. *C.R. Acad. Sci. U.R.S.S.* **40**, 26.

Virtanen, A. I. (1936). The mechanism of the symbiotic nitrogen fixation. *Suomen Kemistilehti*, A, **9**, 69.

Virtanen, A. I. (1938). *Cattle Fodder and Human Nutrition.* Cambridge.

Virtanen, A. I. (1939). Mechanism of symbiotic nitrogen fixation by leguminous plants. *Trans. Third Comm. Int. Soc. Soil Sci.* A, p. 4.

Virtanen, A. I. (1947). The biology and chemistry of nitrogen fixation. *Biol. Rev.* **22**, 239.

Virtanen, A. I. & Arhimo, A. A. (1939*a*). Formation of amino acids in green plants with nitrate as the nitrogen source. *Trans. Third Comm. Int. Soc. Soil Sci.* B, p. 20.

Virtanen, A. I. & Arhimo, A. A. (1939*b*). Oxalacetic acid in the leguminous plants. *Nature, Lond.*, **144**, 36.

Virtanen, A. I., Arhimo, A. A., Sundmann, J. & Jännes, L. (1943). Vorkommen und Bedeutung der Oxalessigsäure in grünen Pflanzen. *J. prakt. Chem.* **162**, 71.

Virtanen, A. I. & Erkama, J. (1938). Enzymatic deamination of aspartic acid. *Nature, Lond.*, **142**, 954.

Virtanen, A. I. & Laine, T. (1937*a*). Formation of β-alanine from aspartic acid through the legume bacteria. *Suomen Kemistilehti*, B, **10**, 2.

Virtanen, A. I. & Laine, T. (1937*b*). N fixation by excised root nodules. *Suomen Kemistilehti*, B, **10**, 24.

Virtanen, A. I. & Laine, T. (1937*c*). Excretion of *l*-aspartic acid from the root nodules of leguminous plants. *Suomen Kemistilehti*, B, **10**, 32.

Virtanen, A. I. & Laine, T. (1938). Biological synthesis of amino acids from atmospheric nitrogen. *Nature, Lond.*, **141**, 748.

Virtanen, A. I. & Laine, T. (1939). Investigations on the root nodule bacteria of leguminous plants. *Biochem. J.* **33**, 412.

Virtanen, A. I. & Laine, T. (1941). Über die Umaminierung in grünen Pflanzen. *Biochem. Z.* **308**, 213.

Virtanen, A. I., Laine, T. & Roine, P. (1938). The determination of oxalacetic acid in green plants. *Suomen Kemistilehti*, B, **11**, 25.

Virtanen, A. I., Laine, T. & von Hausen, S. (1936). Excretion of aminoacids from the root nodules and their chemical nature. *Suomen Kemistilehti*, B, **9**, 1.

Virtanen, A. I. & Linkola, H. (1946). Organic nitrogen compounds as nitrogen nutrition for higher plants. *Nature, Lond.*, **158**, 515.

Virtanen, A. I., Linkola, H., Hakala, M. & Rautanen, N. (1946). Glutamic acid among the excretion products of leguminous root nodules. *Suomen Kemistilehti*, B, **19**, 83.

Virtanen, A. I., Rintala, P. & Laine, T. (1938). Decarboxylation of aspartic and glutamic acids. *Nature, Lond.*, **142**, 674.

Virtanen, A. I. & Saastamoinen, S. (1936). Untersuchungen über Stickstoffbindung bei der Erle. *Biochem. Z.* **284**, 72.

Virtanen, A. I., von Hausen, S. & Laine, T. (1937*a*). Investigations of the root nodule bacteria of leguminous plants. 19. Influence of various factors on the excretion of nitrogenous compounds from the nodules. *J. agric. Sci.* **27**, 332.

Virtanen, A. I., von Hausen, S. & Laine, T. (1937*b*). Investigations of the root nodule bacteria of leguminous plants. 20. Excretion of nitrogen in associated cultures of legumes and non-legumes. *J. agric. Sci.* **27**, 584.

Vladescu, I. (1934). Assimilation of mineral and organic substances during the development of the tobacco plant. *Bul. cultivarei fermentarei tutunului*, **23**, 231, 359 (quoted from Vickery *et al.* (1935) and *Chem. Abstr.* **29**, 2204, 2999).

Vladimirov, A. V. (1939). Ammonium and nitrate supplies compared with regard to their effect on biochemical processes in the leaves of *Nicotiana rustica*. *C.R. Acad. Sci. U.R.S.S.* **23**, 699.

Vladimirov, A. V. (1945). Influence of nitrogen source on the formation of oxidised and reduced organic compounds in plants. *Soil Sci.* **60**, 265.

Vlasyuk, P. A. (1940*a*) Einfluss des Mikroelementes Mangan auf die Ausnutzung der Ammoniak- und Nitratform des Stickstoffes durch Verpflanzung der Zuckerrübe. *C.R. Acad. Sci. U.R.S.S.* **28**, 181.

Vlasyuk, P. A. (1940*b*). Über die Bedeutung des Mangans bei der Ausnutzung der Ammoniak- und Nitratform der Stickstoffnährung für die Wasserkultur der Zuckerrübe. *C.R. Acad. Sci. U.R.S.S.* **28**, 184.

von Faber, F. C. (1912). Das erbliche Zusammenleben von Bakterien und tropischen Pflanzen. *Jb. wiss. Bot.* **51**, 285.

von Körösy, K. (1913). Über die Chlorophyllassimilation. *Hoppe-Seyl. Z.* **86**, 368.

von Plotho, O. (1941). Die Synthese der Knöllchen an den Wurzeln der Erle. *Arch. Mikrobiol.* **12**, 1.

Vsevolozhskaya, G. K. (1946). Influence of nitrogen nutrition upon acceleration of flowering and maturation in *Hibiscus cannabinus* (kenaph) and jute. *C.R. Acad. Sci. U.R.S.S.* **53**, 847.

Wadleigh, C. H. (1944). Growth status of the cotton plant as influenced by the supply of nitrogen. *Bull. Ark. agric. Exp. Sta.* no. 446.

Wadleigh, C. H. & Shive, J. W. (1939). Organic acid content of corn plants as influenced by pH of substrate and form of nitrogen supplied. *Amer. J. Bot.* **26**, 244.

Wager, H. G. (1938). Growth and survival of plants in the Arctic. *J. Ecol.* **26**, 390.

Walker, J. C. & Foster, R. E. (1946). Plant nutrition in relation to disease development. *Amer. J. Bot.* **33**, 259.

Walker, J. C. & Hooker, W. J. (1945*a*). Plant nutrition in relation to disease development. I. Cabbage yellows. *Amer. J. Bot.* **32**, 314.

Walker, J. C. & Hooker, W. J. (1945*b*). Plant nutrition in relation to disease development. II. Cabbage clubroot. *Amer. J. Bot.* **32**, 487.

Walkley, J. (1940). Protein synthesis in mature and senescent leaves of barley. *New Phytol.* **39**, 362.

Walkley, J. & Petrie, A. H. K. (1941). Studies on the nitrogen metabolism of plants. IV. On the changing nature of the relation between proteins and amino acids. *Ann. Bot., Lond.*, N.S. **5**, 661.

Wallebroek, J. C. (1940). Alkaloid and nitrogen metabolism in the germination of *Lupinus luteus*. Diss., Amsterdam (quoted from *Biol. Abstr.* **21**, entry 12,622 (1947)).

Warburg, O. & Negelein, E. (1920). Über die Reduktion der Salpetersäure in grünen Zellen. *Biochem. Z.* **110**, 66.

Ware, L. M. & Johnson, W. A. (1944). Nitrogen requirements of different groups of vegetables. *Proc. Amer. Soc. hort. Sci.* **44**, 343.

Wasteneys, H. & Borsook, H. (1930). The enzymatic synthesis of protein. *Physiol. Rev.* **10**, 110.

Watson, R. & Petrie, A. H. K. (1940). Physiological ontogeny in the tobacco plant. 4. The drift in nitrogen content of the parts in relation to phosphorus supply and topping, with an analysis of the determination of ontogenetic changes. *Aust. J. exp. Biol. med. Sci.* **18**, 313.

Weier, E. (1938). The structure of the chloroplast. *Bot. Rev.* **4**, 497.

Weil-Malherbe, H. (1938). Observations on tissue glycolysis. *Biochem. J.* **32**, 2257.

White, H. L. (1937). The interaction of factors in the growth of *Lemna*. XI. The interaction of nitrogen and light intensity in relation to growth and assimilation. *Ann. Bot., Lond.*, N.S. **1**, 622.

White, H. L. & Templeman, W. G. (1937). The interaction of factors in the growth of *Lemna*. X. The interaction of nitrogen and light intensity in relation to respiration. *Ann. Bot., Lond.*, N.S. **1**, 191.

White, P. R. (1939). Glycine in the nutrition of excised tomato roots. *Plant Physiol.* **14**, 527.

Whitehead, E. I., Olson, O. E. & Moxon, A. L. (1944). Some observations on the nitrate content of oat plants. *Proc. S. Dak. Acad. Sci.* **24**, 61.

Wieland, H. & Rosenthal, C. (1943). Weitere Versuche über den biologischen Abbau der Essigsäure. *Liebigs Ann.* **554**, 241.

Williams, C. H. & Hines, H. J. G. (1939). Toxicity of *Salvia reflexa*. *Nature, Lond.*, **144**, 118.

Williams, R. D. (1939). Genetics of cyanogenesis in white clover (*Trifolium repens*). *J. Genet.* **38**, 357.

Williams, R. F. (1938). Physiological ontogeny in plants and its relation to nutrition. 4. The effect of phosphorus supply on the total-, protein- and soluble-nitrogen contents, and water content of the leaves and other plant parts. *Aust. J. exp. Biol. med. Sci.* **16**, 65.

Williams, R. F. (1946). The physiology of plant growth with special reference to the concept of net assimilation rate. *Ann. Bot., Lond.*, N.S. **10**, 41.

Wilson, B. D. (1921). Nitrogen in the rainwater at Ithaca, N.Y. *Soil Sci.* **11**, 101.

Wilson, J. B. & Wilson, P. W. (1942). Biotin as a growth factor for *Rhizobia*. *J. Bact.* **43**, 329.

Wilson, J. B. & Wilson, P. W. (1943). Action of inhibitors on hydrogenase in *Azotobacter*. *J. gen. Physiol.* **26**, 277.

Wilson, P. W. (1940). *The Biochemistry of Symbiotic Nitrogen Fixation.* Madison, Wisconsin.

Wilson, P. W. & Burris, R. H. (1944). Carbon monoxide as an inhibitor of nitrogen fixation by the alga *Nostoc muscorum*. *J. Bact.* **47**, 410. (Abstract only.)

Wilson, P. W. & Burris, R. H. (1947). The mechanism of biological nitrogen fixation. *Bact. Rev.* **11**, 41.

Wilson, P. W., Burris, R. H. & Coffee, W. B. (1943). Hydrogenase and symbiotic nitrogen fixation. *J. biol. Chem.* **147**, 475.

Wilson, P. W. & Burton, J. C. (1938). Excretion of nitrogen by leguminous plants. *J. agric. Sci.* **28**, 307.

Wilson, P. W., Hull, J. F. & Burris, R. H. (1943). Competition between free and combined nitrogen in nutrition of *Azotobacter*. *Proc. nat. Acad. Sci., Wash.*, **29**, 289.

Wilson, P. W. & Umbreit, W. W. (1937). Mechanism of symbiotic nitrogen fixation. III. Hydrogen as a specific inhibitor. *Arch. Mikrobiol.* **8**, 440.

Willstätter, R. & Heubner, W. (1907). Über eine neue Solanaceenbase. *Ber. dtsch. chem. Ges.* **40**, 3869.

Wingard, S. A. (1941). The nature of disease resistance in plants. I. *Bot. Rev.* **7**, 59.

Winks, W. R. (1940). Variation of the prussic acid content of *Sorghum verticilliflorum* at different stages of growth. *Qd agric. J.* **54**, 364.

Winogradsky, S. (1939). Sur la synthèse biogene de l'ammoniac dans le sol et les eaux. *C.R. Acad. Sci., Paris*, **209**, 616.

Wittwer, S. H., Albrecht, W. A. & Goff, H. R. (1946). Vegetable crops in relation to soil fertility. III. Oxalate content and nitrogen fertilisation. *Food Res.* **11**, 54.

Wittwer, S. H., Schroeder, R. A. & Albrecht, W. A. (1945). Vegetable crops in relation to soil fertility. II. Vitamin C and nitrogen fertilizers. *Soil Sci.* **59**, 329.

Wolf, M. J. & Duggar, B. M. (1946). Estimation and physiological role of solanine in the potato. *J. agric. Res.* **73**, 1.

Wood, H. G. (1946). The fixation of carbon dioxide and the interrelationships of the tricarboxylic acid cycle. *Physiol. Rev.* **26**, 198.

Wood, H. G. & Werkman, C. H. (1936). The utilisation of CO$_2$ in the dissimilation of glycerol by the propionic acid bacteria. *Biochem. J.* **30**, 48.

Wood, H. G. & Werkman, C. H. (1938). The utilisation of CO$_2$ by the propionic acid bacteria. *Biochem. J.* **32**, 1262.

Wood, J. G. (1933). The nitrogen metabolism of the leaves of *Atriplex nummularium*. *Aust. J. exp. Biol. med. Sci.* **11**, 237.

Wood, J. G. (1941). Relations between respiration rate and metabolism of carbohydrate, protein and organic acids in leaves. *Aust. J. exp. Biol. med. Sci.* **19**, 313.

Wood, J. G. (1942). Studies on the nitrogen metabolism of plants. VI. Interrelations among respiration rate, carbohydrates, soluble nitrogen compounds and protein in leaves. *Aust. J. exp. Biol. med. Sci.* **20**, 257.

Wood, J. G. (1945). Nitrogenous constituents of plants. *Ann. Rev. Biochem.* **14**, 665.

Wood, J. G. & Barrien, B. S. (1939*a*). Studies on the sulphur metabolism of plants. I. Preliminary investigations on the effects of different external concentrations of sulphate, ammonia and cystine on the amounts of sulphur-containing compounds in leaves. *New Phytol.* **38**, 125.

Wood, J. G. & Barrien, B. S. (1939*b*). Studies on the sulphur metabolism of plants. III. On changes in amounts of protein sulphur and sulphate sulphur during starvation. *New Phytol.* **38**, 265.

Wood, J. G. & Cruickshank, D. H. (1944). The metabolism of starving leaves. 5. Changes in amount of some amino acids during starvation of grass leaves; and their bearing on the nature of the relationship between proteins and amino acids. *Aust. J. exp. Biol. med. Sci.* **22**, 111.

Wood, J. G., Cruickshank, D. H. & Kuchel, R. H. (1943). The metabolism of starving leaves. I. Presentation of data; the nature of respiration rate/time curves in air and in nitrogen and the relation to carbohydrates. 2. Changes in amounts of total and chloroplast proteins, chlorophyll, ascorbic acid and soluble nitrogen compounds. 3. Changes in malic and citric acid contents and interrelations of these with soluble nitrogen compounds. *Aust. J. exp. Biol. med. Sci.* **21**, 37.

Wood, J. G., Mercer, F. V. & Pedlow, C. (1944). The metabolism of starving leaves. 4. Respiration rate and metabolism of leaves of kikuyu grass during air-nitrogen transfers. *Aust. J. exp. Biol. med. Sci.* **22**, 37.

Wood, J. G. & Petrie, A. H. K. (1938). Studies on the nitrogen metabolism of plants. II. Interrelations among soluble nitrogen compounds, water and respiration rate. *Ann. Bot., Lond.*, N.S. **2**, 729.

Wood, J. G. & Petrie, A. H. K. (1942). Studies on the nitrogen metabolism of plants. V. The relation of carbohydrate content to protein synthesis in leaves. *Aust. J. exp. Biol. med. Sci.* **20**, 249.

WOOD, J. G. & WOMERSLEY, H. B. S. (1946). Development and metabolism of copper-deficient oat plants. *Aust. J. exp. Biol. med. Sci.* **24**, 79.
WOOD, W. A., GUNSALUS, I. C. & UMBREIT, W. W. (1947). Function of pyridoxine phosphate: resolution and purification of the tryptophanase enzyme of *Escherichia coli*. *J. biol. Chem.* **170**, 313.
WOODMAN, R. M. (1939). Studies in the nutrition of vegetables. The effects of variation in the nitrogen supply on lettuce (var. May King) in sand culture. *Ann. Bot., Lond.*, N.S. **3**, 649.
WOODS, D. D. (1938). The reduction of nitrate to ammonia by *Clostridium welchii*. *Biochem. J.* **32**, 2000.
WOODS, M. W. & DU BUY, H. G. (1941). Synthesis of tobacco mosaic virus protein in relation to leaf chromoprotein and cell metabolism. *Phytopathology*, **31**, 978.
WOODWARD, R. B. & SCHRAMM, C. H. (1947). Synthesis of protein analogs. *J. Amer. chem. Soc.* **69**, 1552.
WYND, F. L. (1942). Comparison of the efficiency of single applications with repeated top dressings of nitrogenous fertilizers in increasing the yield of dry matter, nitrogen and vitamin C (ascorbic acid) of sudan grass. *Plant Physiol.* **17**, 645.
WYND, F. L. (1946). Relations between dry weight, protein and vitamins in leaves of cereals. *Food Res.* **11**, 195.
WYSS, O., BURRIS, R. H. & WILSON, P. W. (1939). Occurrence and significance of oxalacetic acid in plant tissues. *Proc. Soc. exp. Biol., N.Y.*, **40**, 372.
WYSS, O. & WILSON, P. W. (1941). Mechanism of biological nitrogen fixation. VI. Inhibition of *Azotobacter* by hydrogen. *Proc. Nat. Acad. Sci.* **27**, 162.
YAMAGATA, S. (1934). Über den Einfluss der Stickstoffquelle auf den Gaswechsel des Schimmelpilzes. Beiträge zur Physiologie der Nitratassimilation. *Acta Phytochim.* **8**, 117.
YAMAGATA, S. (1940). Über Nitratreduktase und die 'Nitritreduktase', ein neues Enzym, von *Bacillus pyocyaneus*. Untersuchungen über die biologischen Reduktionen. II. *Acta Phytochim.* **11**, 1.
YAMAGUCHI, S. (1930). Studies on the resorption of urea by root of *Zea mays* seedlings in sterile culture. *J. Fac. Sci. Hokkaido Univ.*, Ser. 5, Bot., **1**, 37.
YEMM, E. W. (1937). Respiration of barley plants. III. Protein catabolism in starving leaves. *Proc. roy. Soc.* B, **123**, 243.
YEMM, E. W. (1947). Personal communication.
ZELENIN, M. M. (1939). Quantitative spectrographic determination of thiourea in fungal extracts. *Bull. Acad. Sci. U.R.S.S.*, Sér. Biol. p. 832 (Russian).
ZHUKOV, N. I. (1939). Inheritance of nicotine and anabasine in interspecific hybrids *Nicotiana rustica* L. × *N. glauca* Grah. *C.R. Acad. Sci. U.R.S.S.* **22**, 116.

Note added in Proof. Tóth (1944, 1946) and his co-workers (Tóth, Wolsky & Bátori, 1942; Tóth, Wolsky & Bátyka, 1944) have demonstrated an active fixation of nitrogen by bacteria living symbiotically in termites, aphids and other insects. The experiments used surviving organ systems from the insects and, in some cases, isolated bacterial cultures. The regularity and intensity of fixation were markedly increased by addition of oxalacetic acid or succinic acid to the culture medium.

REFERENCES

TÓTH, L. (1944). Stickstoffassimilation und das symbiontische System bei *Kalotermes flavicollis* (Isoptera). *Magyar Biol. Kut. Munk.* **16**, 7.
TÓTH, L. (1946). *The biological fixation of atmospheric nitrogen*. Monographs Natural Science, 5. Budapest.
TÓTH, L., WOLSKY, A. & BÁTORI, M. (1942). Stickstoffassimilation aus der Luft bei den Aphiden und bei den Homopteren. *Z. vergl. Physiol.* **30**, 67.
TÓTH, L., WOLKSY, A. & BÁTYKA, E. (1944). Stickstoffassimilation aus der Luft bei den Rhynchoten (Insecta). *Z. vergl. Physiol.* **30**, 300.

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EXPERIMENTAL METHODS AVAILABLE FOR THE STUDY OF THE NITROGEN METABOLISM OF PLANTS

A REVIEW OF SOME RECENT ADVANCES

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In common with other fields of biological inquiry it is of first importance to consider the experimental material and techniques used when attempting to assess the significance of the data of different workers. Furthermore a proper appreciation of the potentialities of new techniques is indispensable in planning future research.

An understanding of the general nitrogen economy of the plant demands studies of the nitrogen partition in the individual organs during development (Street, Kenyon & Watson, 1946*a*) and the recording of data in such a way that a picture on both a fresh and dry-weight basis for each organ and for the plant as a whole can be constructed. Our present knowledge of the general nitrogen economy of the plant is still largely built up on fragmentary data presented in connexion with the study of some more restricted aspect of metabolism and awaits investigation as a separate project carried out under the carefully controlled environmental conditions recently achieved by Went (1943).

Valuable data on the biochemical paths of degradation and synthesis of nitrogenous compounds have been obtained by the use of whole seedlings and of detached and attached leaves, but in view of the anatomical and hence metabolic complexity of these structures, the full interpretation of these data demands a more detailed knowledge of the biochemistry of simpler systems and a more complete picture of the part individual organs and tissues play in the general nitrogen economy. These considerations have prompted the use of such materials as uniform disks of storage parenchyma (Steward, 1932), excised seedling roots (Hoagland, 1944), and excised embryos (Brown, 1946) in recent studies of nitrogen metabolism, and the insistence on the utilization of material of uniform ontogenetic age (Wood, 1945). The development of techniques for the sterile culture of excised root tips (White, 1943) would seem to provide material particularly suited for research into the ability of the root to absorb and assimilate various forms of nitrogen.

Preliminary work with heavy nitrogen (N_{15}) as a tracer for nitrogen metabolism studies with plants has been undertaken by Hevesy, Langstrøm-Lang, Keston & Carsten (1940) and Vickery, Pucher, Schoenheimer & Rittenberg (1940).

The development of the freeze-drying of biological fluids (Greaves & Adair, 1936; Flosdorf & Mudd, 1935; Flosdorf, Stokes & Mudd, 1940) and its application to whole-plant tissue (Neuberger & Sanger, 1942) may overcome the problem of the preservation of plant material without destruction of its labile nitrogenous constituents, and may prove of particular value in studies of plant proteins and of the enzymes involved in nitrogen metabolism.

General schemes for the extraction and partial analysis of the nitrogenous constituents of plant tissues have been put forward by Orcutt & Wilson (1936), Borsook & Dubnoff (1939), Blackman & Templeman (1940), Schlenker (1940, 1943), Steward & Street (1946) and Crook (1946).

The Kjeldahl method for the determination of nitrogen continues to be the subject of many investigations. Pepkowitz, Prince & Bear (1942) and Pepkowitz & Shive (1942) have used perchloric acid to hasten the digestion, but some of the dangers attendant upon its use are discussed by Wicks & Firminger (1942). Chibnall, Rees & Williams (1943) have made a careful study of the conditions required for the accurate determination of the nitrogen of proteins and have recommended a digestion period of at least 8 hr. Miller & Houghton (1945), in a similar study, have stressed that maximum yields of nitrogen from proteins and amino-acids can only be obtained with a digestion period of at least 6 hr., using mercury as catalyst and by carrying out the distillation under carefully standardized conditions. The application of the Kjeldahl method to various different types of compounds is discussed in papers by Modeer (1940), Acree (1941), Bhat (1941), Clark (1941), Johnson (1941) and White & Secor (1946). The reduced iron method of Pucher, Leavenworth & Vickery (1930) for the determination of total nitrogen including nitrates had been adapted to a micro-scale by Moore (1938). Doneen (1932) has described a micro-adaptation of Ranker's (1927) salicylic-acid method. This method, which involves evaporation to dryness under controlled conditions before treatment of extracts with the salicylic-sulphuric acid mixture, was found by Street, Kenyon & Watson (1946*b*) to give higher and more consistent values than the reduced iron method when applied to tissues very high in nitrate content. Blackman & Templeman (1940) have made a careful comparison of values for the total nitrogen of plant tissues obtained by the macro-salicylic and micro-reduced iron method. Umbreit & Bond (1936) have used an alkaline reduction with Devarda's alloy before the acid digestion to obtain a nitrogen value inclusive of nitrate, and Reifer (1939*b*) has used titanous chloride to reduce nitrate prior to digestion. New micro-Kjeldahl distillation units are described by Hoch (1940), Lecoq (1940), Brant & Sievers (1941), Ma & Zuazaga (1942) and Markham (1942). Modifications of the micro-diffusion method are used by Kaweran (1941), Hawes & Skavinski (1942), Prater, Cowles & Straka (1942) and Conway & O'Malley (1942). The use of boric acid as absorbent is adopted in several recent papers (Borsook & Dubnoff, 1939; Wagner, 1940; Hendricks, Thomas, Stout & Tolman, 1942; Ma & Zuazaga, 1942; Taylor & Smith, 1942; Conway & O'Malley, 1942). The application of Nessler's reagent to the determination of Kjeldahl nitrogen is discussed by Gillo (1939), Herter (1940), Hoffman & Osgood (1940), Mason & Rozzell (1940), Mácola & Fazio (1941), Wicks (1941), Wolf (1944*a*), and Kelley, Hunter & Sterges (1946). Russell (1944) has described a colorimetric method for determination of the ammonia using the phenol-hypochlorite reaction.

The micro-aeration apparatus of Sobel, Mayer & Gottfried (1944) and the micro-vacuum distillation apparatus of Archibald (1943) are both well adapted for the determination of the free ammonia of plant tissues containing glutamine or other labile nitrogen compounds.

The determination of nitrate by reduction with Devarda's alloy in alkaline solution (Sessions & Shive, 1928; Sessions, 1930; Gerdel, 1932) has been the subject of papers by Shrikhande (1941) and Kieselbach (1944). Kieselbach (1944) describes a special apparatus for the nitrate reduction and for recovery of the ammonia formed. The Devarda method has given satisfactory results in the hands of Orcutt & Wilson (1936), Borsook & Dubnoff (1939), Blackman & Templeman (1940) and Street *et al.* (1946*a*, *b*). Reifer (1939*a*) has proposed reduction of the nitrate of plant extracts by titanous chloride. Vickery & Pucher (1929) determined nitric acid nitrogen by ether extraction followed by a reduction with reduced iron. The phenol-disulphonic acid colorimetric method for determination of nitrate (Gilbert, 1926) has been studied by Frear (1930) and Ashton (1935) and adopted as the method of choice by Roller & McKaig (1939) and Schlenker (1943). Blackman & Templeman (1940), using the method of Ashton, obtained low values due to loss of nitrate during the clearing procedure, but direct determinations without clearing gave results agreeing with those obtained by the Devarda method. Wolf (1943, 1944*b*) has described a colorimetric method for nitrate based on the use of a solution of brucine in concentrated sulphuric acid. The possibility of a polarographic method for the determination of nitrate is discussed by Scott & Bambach (1942).

Nitrite normally occurs in only very low concentrations in plant tissues, and therefore its quantitative estimation is one of considerable difficulty. Shinn (1941) has published a modification of the method of Bratton, Marshall, Babbitt & Hendrickson (1939), and Rider & Mellon (1946) have made a careful study of the errors in the Official Method of the Association of Official Agricultural Chemists (1940). Wolf (1944*b*) has developed a colorimetric method using a dimethylaniline reagent. Steward & Street (1947) have discussed some of the difficulties encountered in determining nitrite and hydroxylamine in plant extracts.

Most workers, until recently, have determined the amino-nitrogen of plant extracts by the Van Slyke nitrous acid method (Van Slyke, 1912, 1913, 1915). Fictitious values for Van Slyke amino-nitrogen given by non-nitrogenous compounds have been reported by Clarke & Inouye (1930), Rahn (1932), Stuart (1935), Kendrick & Hanke (1940) and Carter & Dickman (1943). It has also been shown that ammonia (Van Slyke, 1912), urea (Plimmer, 1925) and various peptides (Thierfelder & von Cramm, 1919) react with nitrous acid yielding nitrogen, and that lysine, cystine, glycine (Schmidt, 1938) and glutamine (Vickery, Pucher, Clark, Chibnall & Westall, 1935) give values deviating from theoretical. Fraenkel-Conrat (1943) has shown that tyrosine and other phenolic compounds give values in excess of theoretical when the reaction chamber is exposed to light. The Van Slyke method is not only beset with these complications but it determines the nitrogen of all amino-groups not involved in peptide linkages and, under the acid conditions employed, peptides are likely to suffer hydrolysis. The values obtained when this method is applied to crude plant extract cannot be regarded as an accurate measure of the amino-nitrogen of the free amino-acids. The more recent gasometric ninhydrin method (Van Slyke & Dillon, 1936, 1938; Van Slyke, Dillon, MacFadyen & Hamilton, 1941) is specific for free amino-acids in that it required the presence, in the free unconjugated state, of both the carboxyl and the neighbouring NH_2 or $NH\text{-}CH_2$ group. Volatile aldehydes can interfere with the method, but this difficulty is easily surmounted (Schott, Rockland & Dunn, 1944; Hamilton & Van Slyke, 1946). There are now available procedures for determination of the ninhydrin value by titration either of the carbon dioxide (Van Slyke, MacFadyen & Hamilton, 1941; Christensen, West & Dimick, 1941) or of the ammonia produced (Schlenker, 1943; MacFadyen, 1944; Sobel, Hirschman & Besman, 1945). The ninhydrin method should now replace the nitrous acid method for determination of the free amino-acid nitrogen of plant extracts.

Determination of amino-nitrogen by formol titration is the subject of a recent paper by Sisco, Cunningham & Kirk (1941). Formol-titration has been recently used by various workers for the determination of amino-nitrogen in plant extracts (Richards & Templeman, 1936; Borsook & Dubnoff, 1939; Blackman & Templeman, 1940; Brown, 1946).

Convenient colorimetric methods for the determination of amino-nitrogen are described by Frame, Russell & Wilhelmi (1943) and Russell (1944).

As a result of the development of new techniques it should now be possible to undertake the quantitative determination of individual amino-acids in metabolic studies, and a move in this direction has already been made by Wood & Cruikshank (1944). Procedures for the estimation of individual amino-acids on a micro-scale by partition chromotography are described by Gordon, Martin & Synge (1943, 1944) and Sims (1945). Partition chromotography on paper devised originally by Consden, Gordon & Martin (1944) and first applied to plant material by La Cour & Drew (1947) has now, in the hands of Dent, Stepka & Steward (1947), yielded precise and interesting results. The method has proved capable of the separate detection and semi-quantitative estimation of nineteen amino-acids and of the amides asparagine and glutamine in an alcoholic extract of potato-tuber tissue. Woiwod (1948) has also successfully applied the Pope & Stevens copper phosphate method (1939) to the estimation of micro-amounts of amino-acids separated by paper partition chromatography.* The work of Zittle & Eldred (1944) and Gale (1945) on the estimation of amino-acids by the use of the specific bacterial decarboxylases may also be valuable in this connexion. The recent methods of biological assay of individual amino-acids not only makes possible inquiry into the pathways of amino-acid synthesis in micro-organisms but should prove valuable for the determination of individual amino-acids in plant extracts (Shankman, Dunn & Ruben, 1943*a*, *b*; Kuiken, Norman, Lyman, Hale & Blotter, 1943; Dunn, Camien, Rockland, Shankman & Goldberg, 1944; Dunn, Camien, Shankman, Frankl & Rockland, 1944; Dunn, Camien, Shankman & Rockland, 1945; Dunn, Schott, Frankl & Rockland, 1945; Dunn, Shankman & Camien, 1945*a*, *b*; Dunn, Camien, Shankman & Block, 1946; Dunn, Shankman, Camien & Block, 1946; Greene & Black, 1944; Hegsted, 1944; McMahan & Snell, 1944; Ryan & Brand, 1944; Schweigert, McIntire, Elvehjem & Strong, 1944; Doermann, 1945; Hac & Snell, 1945; Hac, Snell & Williams, 1945; Hier & Bergeim, 1945, 1946; Hier, Graham, Freides & Klein, 1945; Lewis & Olcutt, 1945; Lyman, Kuiken, Blotter & Hale, 1945; Stokes & Gunness, 1945; Stokes, Gunness, Dwyer & Caswell, 1945; Wooley & Sebrell, 1945; Greenhut, Schweigert & Elvehjem, 1946; Gunness, Dwyer & Stokes, 1946; Riesen, Schweigert & Elvehjem, 1946). Chemical methods for the determination of individual amino-acids, excellently summarized by Block & Bolling (1940, 1945), may in some cases be applicable in plant studies.

The total amide nitrogen of plant extracts is readily determined by the method of Vickery, Pucher & Leavenworth (1935) using 6 N-sulphuric acid as the hydrolysing agent. Vickery, Pucher, Clark, Chibnall & Westall (1935) have developed a hydrolytic method for the determination of glutamine. The extracts used should be alcohol free, as alcohol markedly inhibits glutamine hydrolysis under the conditions employed (Steward & Street, 1946). Vickery, Pucher, Wakeman & Leavenworth (1939), applying this hydrolytic method for glutamine to the blade and petiole of rhubarb, obtained in some cases negative values for asparagine, showing that the glutamine values obtained were too high. Pucher & Vickery (1940) have now described a more specific method which involves extraction of the pyrrolidone carboxylic acid resulting from glutamine hydrolysis. This method, though tedious, overcomes interference from urea and allantoin and is to be recommended when these compounds are present in appreciable amount or when the amount of glutamine is small compared with that of asparagine. Neuberger & Sanger (1942) adapted the Van Slyke-Dillon ninhydrin method to the determination of glutamine, and this has been the subject of a detailed study by Hamilton (1945). Archibald (1944*a*, *b*) has studied the preparation and assay of glutaminase from dog kidney and has described a procedure for the enzymatic determination of glutamine. This method should be of value in checking, for any given experimental material, the accuracy of the simpler hydrolytic and ninhydrin procedures.

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* See also: Naftalin, L. (1948). Quantitative chromatographic estimation of α-amino-acids. *Nature, Lond.*, **161**, 763.

The term 'basic nitrogen' was given by Umbreit & Wilson (1936) and Orcutt & Wilson (1936) to the nitrogen precipitated by treatment of deproteinized plant extracts with phosphotungstic acid. The amino-nitrogen of this precipitate was termed 'basic amino-nitrogen'. The nature of the phosphotungstic acid precipitate will vary considerably according to the constituents present, and even with closely similar extracts comparable results will only be obtained by very carefully standardizing the precipitation conditions. The determination of 'non-amino basic nitrogen' has proved, in general, of little value in view of its uncertain composition. The determination of 'basic amino-nitrogen' has been used to follow changes in proportionality among the free amino-acids (Viets, Jr., Moxon & Whitehead, 1946), but since the basic amino-acids, histidine, arginine and lysine, are amongst those most readily determined individually, this would seem to be of greater value.

Orcutt & Wilson (1936) and Borsook & Dubnoff (1939) have determined 'peptide nitrogen' on the basis of the increase in amino-nitrogen resulting from treatment of deproteinized plant extracts with a mixture of proteolytic enzymes prepared from *Aspergillus wentii*. Vickery *et al.* (1939) and Viets *et al.* (1946) have determined 'peptide nitrogen' by the increase in amino-nitrogen resulting from acid hydrolysis, a method which, however, results in considerable humin formation. Sideris & Young (1946) have termed as 'proteose-peptone' the nitrogen precipitated from deproteinized extracts by a lead acetate reagent, and as 'peptide' the nitrogen precipitated from the filtrate by treatment with a mercuric acetate reagent. The composition of such precipitates is, however, uncertain and very variable.

Exhaustive extraction of plant tissues with cold water or 70% alcohol leaves a residue, the nitrogen content of which, determined either directly or by difference, has been termed 'insoluble protein'. 'Total protein' has been determined in a similar manner by extraction of the tissues with water or alcohol at 80° C., so that a deproteinized filtrate is obtained. Prolonged extraction at high temperature yields extracts in which decomposition of labile nitrogenous compounds has occurred. It is therefore usual to prepare extracts in the cold, and then deproteinize and determine 'total protein' as the difference between total nitrogen of the tissue and total nitrogen of the deproteinized extract ('total non-protein' nitrogen). The various procedures available for precipitation of protein from plant extracts are discussed by Steward & Street (1946). Hanson, Barrien & Wood (1941) and Wood, Cruikshank & Kuchel (1943) have described and carried out in their experiments determinations of 'chloroplast protein'. As a result of recent work, it is now possible to obtain reasonably representative samples of the whole proteins of leaves (Lugg & Weller, 1944), of chloroplast protein (Menke, 1938*a*, *b*, 1940; Granick, 1938; Neish, 1939; Smith, 1940, 1941; Hanson, 1941; Galston, 1943) and composite mixtures of cytoplasmic proteins or of vacuolar proteins from leaves (Chibnall, 1939). By the application of new techniques Wildman & Bonner (1947) effected a separation of the cytoplasmic proteins of spinach leaves into two fractions. The first fraction, constituting 75–80% of the total cytoplasmic proteins, appears to be electrophoretically homogeneous. Bound auxin and phosphatase activity appear to be integral properties of this fraction. The second smaller fraction, with which were associated all the other enzymatic activities of the cytoplasm, consists of a mixture of proteins as demonstrated by analysis in a Tiselius moving-boundary apparatus.

The reineckate method for the determination of choline has been the subject of papers by Jacobi, Baumann & Meek (1941), Engel (1942), Thornton & Broome (1942), Marenzi & Cardini (1943), Enterman, Taurog & Chaikoff (1944), Glick (1944), Enterman & Chaikoff (1945) and Wurzler & Meserve (1945). The application of these methods directly to crude plant extracts, however, does not give satisfactory results. Strack & Schwaneberg (1937) and Neuberger & Sanger (1942) have pointed out that, whilst all basic substances give reineckates relatively insoluble at acid or sometimes even at neutral or mildly alkaline reactions, only quaternary bases without a carboxyl group, such as choline and methylpyridinium chloride, give reineckates insoluble at pH 12–13. Treatment of crude plant extracts with reineckate at such a highly alkaline reaction, however, usually gives precipitates intractable to filtration so that decomposition occurs during this process. Street, Kenyon & Watson (1946*c*) have studied the earlier method of Lintzel & Monasterio (1931), which is based on the almost quantitative formation of trimethylamine when choline solutions are treated with dilute alkaline permanganate under carefully controlled conditions. It was shown that this method, though inapplicable to the direct determination of choline in crude plant extracts, could, when combined with a preliminary reinecke salt precipitation, form the basis of a rapid and convenient method. Horowitz & Beadle (1943), Luecke & Pearson (1944*a*) and Hodson (1945) have described microbiological methods for choline determination using a choline-less strain of *Neurospora*, and Luecke & Pearson (1944*b*) have made a comparison of their microbiological method with the reineckate methods of Engel (1942) and Marenzi & Cardini (1943). Artom (1945) has described a method for the determination of ethanolamine.

The determination of betaine, particularly when present in small concentration and along with choline, is a matter of considerable difficulty. Reifer (1941) has published a micro-method for the determination in plant extracts of choline and betaine by precipitation as periodide. There are, however, technical difficulties in handling the periodide precipitate and of achieving quantitative separation of the two bases by regulation of the pH of precipitation. Fox, McNiel & Field (1943) have described a method for determination of trigonelline, and a number of recent papers (Arnold, Schieffler & Lipsius, 1941; Dann & Handler, 1941; Melnick, Oser & Siegel, 1941; Waismann & Elvehjem, 1941; Allinson, 1943; Wang & Kodicek, 1943; Martinek, Kirch & Webster, 1943; Teiri & Shimer, 1944) are devoted to methods for the estimation of nicotinic acid.

Methods for the determination of purines are described by Kerr (1940), Hutchings & Fiske (1941), Kalckar (1945) and Kerr & Seraidarian (1945); for uric acid by Blauch & Koch (1939) and Fearon (1944); for allantoin by Florkin & Duchateau (1940), Archibald

(1944*c*), and Christman, Foster & Esterer (1944); and for nucleic acids by Schmidt & Thannhauser (1945), Schneider (1945*a*, *b*) and Williams (1945).

The standard methods of alkaloidal assay are long and tedious processes, and often demand the extraction of a considerable weight of tissue. On both these grounds they are unsuitable for routine use in metabolic studies. The method for the assay of the total alkaloids of solanaceous plants recently described by Roberts & James (1947) marks a new approach and has resulted in a rapid procedure requiring for each estimation only 1 g. of dried tissue. The method of partition chromatography would appear to offer possibilities of alkaloidal estimation using still smaller quantities of plant material.

REFERENCES

ACREE, F., JR. (1941). Collaborative report on the micro- and semimicro-Kjeldahl nitrogen method. *J. Ass. off. agric. Chem., Wash.*, **24**, 648.

ALLINSON, M. J. C. (1943). A specific enzymatic method for the determination of nicotinic acid in blood. *J. biol. Chem.* **147**, 785.

ARCHIBALD, R. M. (1943). Quantitative micro-determination of ammonia in presence of glutamine and other labile substances. *J. biol. Chem.* **151**, 141.

ARCHIBALD, R. M. (1944*a*). The enzymatic determination of glutamine. *J. biol. Chem.* **154**, 643.

ARCHIBALD, R. M. (1944*b*). Preparation and assay of glutaminase for glutamine determinations. *J. biol. Chem.* **154**, 657.

ARCHIBALD, R. M. (1944*c*). Determination of citrulline and allantoin and demonstration of citrulline in blood plasma. *J. biol. Chem.* **156**, 121.

ARNOLD, A., SCHIEFFLER, C. B. & LIPSIUS, S. T. (1941). Chemical determination of nicotinic acid. *Industr. Engng Chem.* (Anal. ed.), **13**, 62.

ARTOM, C. (1945). A quantitative method for ethanolamine and serine as a basis for the determination of phosphatidyl ethanolamine and phosphatidyl serine in tissue. *J. biol. Chem.* **157**, 585.

ASHTON, F. L. (1935). A rapid colorimetric method for determining nitrate nitrogen in grass. *J. Soc. chem. Ind., Lond.*, **54**, 389.

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS (1940). *Official and Tentative Methods of Analysis*, 5th ed. Washington.

BHAT, R. V. (1941). Adaptation of the micro-Kjeldahl method to the estimation of nitrogen containing nitro- and azo-groups. *Proc. Ind. Acad. Sci.* **13**, A, 269.

BLACKMAN, G. E. & TEMPLEMAN, W. G. (1940). The interaction of light intensity and nitrogen supply in the growth and metabolism of grasses and clover (*Trifolium repens*). III. Analytical methods for the estimation of some nitrogen and carbohydrate fractions. *Ann. Bot., Lond.*, N.S. **4**, 119.

BLAUCH, M. B. & KOCH, F. C. (1939). A new method for the determination of uric acid in blood, with uricase. *J. biol. Chem.* **130**, 443.

BLOCK, R. J. & BOLLING, D. (1940). *The Determination of the Amino-acids.* Minneapolis: Burgess Publishing Co.

BLOCK, R. J. & BOLLING, D. (1945). *The Amino-acid Composition of Proteins and Foods.* Illinois: C. C. Thomas.

BORSOOK, H. & DUBNOFF, J. W. (1939). Methods for the determination of submicroquantities of total nitrogen, ammonia, amino nitrogen, amides, peptides, adenylic acid and nitrates. *J. biol. Chem.* **131**, 165.

BRANT, J. H. & SIEVERS, D. C. (1941). Semi-micro and micro-Kjeldahl steam distillation unit. *Industr. Engng Chem.* (Anal. ed.), **13**, 133.

BRATTON, A. C., MARSHALL, E. K., JR., BABBITT, D. & HENDRICKSON, A. R. (1939). A new coupling component for sulfanilamide determination. *J. biol. Chem.* **128**, 537.

BROWN, R. (1946). Studies on germination and seedling growth. III. Early growth in relation to certain aspects of nitrogen metabolism in the seedling of barley. *Ann. Bot., Lond.*, N.S., **10**, 73.

CARTER, H. E. & DICKMAN, S. R. (1943). Anomalous amino-nitrogen values. *J. biol. Chem.* **149**, 571.

CHIBNALL, A. C. (1939). *Protein Metabolism in the Plant.* Yale Univ. Press, New Haven.

CHIBNALL, A. C., REES, M. W. & WILLIAMS, E. F. (1943). The total nitrogen content of egg albumin and other proteins. *Biochem. J.* **37**, 354.

CHRISTENSEN, B. E., WEST, E. E. & DIMICK, K. P. (1941). A simple apparatus and procedure for determination of amino-acids by the ninhydrin reaction. *J. biol. Chem.* **137**, 735.

CHRISTMAN, A. A., FOSTER, P. W. & ESTERER, M. B. (1944). The allantoin content of blood. *J. biol. Chem.* **155**, 161.

CLARK, E. P. (1941). Report on microchemical methods. Kjeldahl nitrogen method. *J. Ass. off. agric. Chem., Wash.*, **24**, 641.

Clarke, H. T. & Inouye, J. M. (1930). Some observations on the action of alkali upon cystine and cysteine. *J. biol. Chem.* **89**, 399.

Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). Qualitative analysis of proteins: a partition chromatographic method using paper. *Biochem. J.* **38**, 224.

Conway, E. J. & O'Malley, E. (1942). Microdiffusion methods. Ammonia and urea using buffered absorbents (revised methods for ranges greater than 10 μg. N). *Biochem. J.* **36**, 655.

Crook, E. M. (1946). The extraction of nitrogenous materials from green leaves. *Biochem. J.* **40**, 197.

Dann, W. J. & Handler, P. (1941). The quantitative estimation of nicotinic acid in animal tissues. *J. biol. Chem.* **140**, 201.

Dent, C. E., Stepka, W. & Steward, F. C. (1947). Detection of the free amino-acids of plant cells by partition chromatography. *Nature, Lond.*, **160**, 682.

Doermann, A. H. (1945). A bioassay for lysine by use of a mutant of *Neurospora*. *J. biol. Chem.* **160**, 95.

Doneen, L. D. (1932). A micro-method for nitrogen in plant material. *Plant Physiol.* **7**, 717.

Dunn, M. S., Camien, M. N., Rockland, L. B., Shankman, S. & Goldberg, S. C. (1944). Investigations of amino-acids, peptides and proteins. XVII. The determination of glutamic acid in protein hydrolysates by a microbiological method. *J. biol. Chem.* **155**, 591.

Dunn, M. S., Camien, M. N., Shankman, S. & Block, H. (1946). Investigations of amino-acids, peptides and proteins. XXVI. The determination of methionine in protein hydrolysates with *Lactobacillus Fermenti* 36. *J. biol. Chem.* **163**, 577.

Dunn, M. S., Camien, M. N., Shankman, S., Frankl, W. & Rockland, L. B. (1944). Investigations of amino-acids, peptides and proteins. XIX. The determination of lysine in protein hydrolysates by a microbiological method. *J. biol. Chem.* **156**, 715.

Dunn, M. S., Camien, M. N., Shankman, S. & Rockland, L. B. (1945). Investigations of amino-acids, peptides and proteins. XXI. The determination of histidine in protein hydrolysates with *Leuconostoc mesenteroides*, P60. *J. biol. Chem.* **159**, 653.

Dunn, M. S., Schott, H. F., Frankl, W. & Rockland, L. B. (1945). Investigations of amino-acids, peptides and proteins. XX. The determination of apparent free tryptophane in blood by a microbiological method. *J. biol. Chem.* **157**, 387.

Dunn, M. S., Shankman, S. & Camien, M. N. (1945*a*). Investigations of amino-acids, peptides and proteins. XXII. The determination of phenylalanine in protein hydrolysates with *Leuconostoc mesenteroides*, P60 and *Lactobacillus casei*. *J. biol. Chem.* **161**, 643.

Dunn, M. S., Shankman, S. & Camien, M. N. (1945*b*). Investigations of amino-acids, peptides and proteins. XXV. The determination of histidine in protein hydrolysates with *Lactobacillus Fermenti* 36. *J. biol. Chem.* **161**, 699.

Dunn, M. S., Shankman, S., Camien, M. N. & Block, H. (1946). Investigations of amino-acids, peptides and proteins. XXVII. The determination of threonine in protein hydrolysates with *Lactobacillus Fermenti* 36. *J. biol. Chem.* **163**, 589.

Engel, R. W. (1942). Modified methods for the chemical and biological determination of choline. *J. biol. Chem.* **144**, 701.

Enterman, C. & Chaikoff, I. L. (1945). On the determination of choline in the liver and plasma of the dog. *J. biol. Chem.* **160**, 377.

Enterman, C., Taurog, A. & Chaikoff, I. L. (1944). The determination of choline in phospholipids. *J. biol. Chem.* **155**, 13.

Fearon, W. R. (1944). The detection and estimation of uric acid by 2 : 6-dichloroquinone-chloromide. *Biochem. J.* **38**, 399.

Florkin, M. & Duchateau, G. (1940). Dosage photométrique de l'Allantoine avec hydrolyse alcaline. Essai d'application à l'urine. *C.R. Soc. Biol., Paris*, **134**, 266.

Flosdorf, E. W. & Mudd, S. (1935). Procedure and apparatus for preservation in 'lyophile' form of serum and other biological substances. *J. Immunol.* **29**, 389.

Flosdorf, E. W., Stokes, F. J. & Mudd, S. (1940). The Desivac process for drying from the frozen state. *J. Amer. med. Ass.* **115**, 1095.

Fox, S. W., McNeil, E. W. & Field, H., Jr. (1943). A simple photoelometric estimation of trigonelline. *J. biol. Chem.* **147**, 645.

Fraenkel-Conrat, H. (1943). The effect of light in the Van Slyke method for the determination of amino-groups. *J. biol. Chem.* **148**, 453.

Frame, E. G., Russell, J. A. & Wilhelmi, A. E. (1943). The colorimetric estimation of amino-nitrogen in blood. *J. biol. Chem.* **149**, 255.

Frear, D. E. (1930). Estimation of nitrate nitrogen in plant juice: a study of the expression and clarification of the juice. *Plant Physiol.* **3**, 359.

Gale, E. F. (1945). Studies in bacterial amino-acid decarboxylases. V. The use of specific decarboxylase preparations in the estimation of amino-acids and in protein analyses. *Biochem. J.* **39**, 46.

Galston, A. W. (1943). The isolation, agglutination and nitrogen analysis of intact oat chloroplasts. *Amer. J. Bot.* **30**, 331.

Gerdel, R. W. (1932). Determination of the inorganic nitrogen in the corn plant by the expressed sap method. *Plant Physiol.* **7**, 517.

Gilbert, B. E. (1926). The adaptation of certain colorimetric methods to the estimation of nitrates, phosphates and potassium in plant solutions. *Plant Physiol.* **1**, 191.

Gillo, L. (1939). Études experimentale sur la stabilité de la réaction de Nessler et ses applications au dosage photométrique de l'azote. *Bull. Soc. Chim. biol., Paris*, **21**, 1117.

Glick, D. (1944). Concerning the reineckate method for the determination of choline. *J. biol. Chem.* **156**, 643.

Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1943). Partition chromatography in the study of protein constituents. *Biochem. J.* **37**, 79.

Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1944). Technical notes on partition chromatography of acet-amino acids with silica gel. *Biochem. J.* **38**, 65.

Granick, S. (1938). Quantitative isolation of chloroplasts from higher plants. *Amer. J. Bot.* **25**, 558.

Greaves, R. I. N. & Adair, M. E. (1936). Simple method for preservation of sera by desiccation in frozen state without use of refrigerants. *J. Hyg., Camb.*, **36**, 507.

Greene, R. D. & Black, A. (1944). The microbiological assay of tryptophane in proteins and food. *J. biol. Chem.* **155**, 1.

Greenhut, I. T., Schweigert, B. S. & Elvehjem, C. A. (1946). The amino-acid requirements of *Streptococcus faecalis* and the use of this organism for the determination of threonine in natural products. *J. biol. Chem.* **162**, 69.

Gunness, M., Dwyer, I. M. & Stokes, J. L. (1946). Microbiological methods for the determination of amino-acids. *J. biol. Chem.* **163**, 159.

Hac, L. R. & Snell, E. E. (1945). The microbiological determination of amino-acids. III. Assay of aspartic acid with *Leuconostoc mesenteroides*. *J. biol. Chem.* **159**, 291.

Hac, L. R., Snell, E. E. & Williams, R. J. (1945). The microbiological determination of amino-acids. II. Assay and utilisation of glutamic acid and glutamine by *Lactobacillus arabinosus*. *J. biol. Chem.* **159**, 273.

Hamilton, P. B. (1945). Gasometric determination of glutamine amino acid carboxyl nitrogen in plasma and tissue filtrates by the ninhydrin-carbon dioxide method. *J. biol. Chem.* **158**, 375.

Hamilton, P. B. & Van Slyke, D. D. (1946). The effects of the volatile aldehydes formed on the accuracy of the manometric ninhydrin-carbon dioxide method in analysis of certain α-amino-acids. *J. biol. Chem.* **164**, 249.

Hanson, E. A. (1941). A note on the metabolism of chloroplast protein. *Aust. J. exp. Biol. med. Sci.* **19**, 157.

Hanson, E. A., Barrien, B. S. & Wood, J. G. (1941). Relations between protein-nitrogen, protein-sulphur and chlorophyll in leaves of Sudan grass. *Aust. J. exp. Biol. med. Sci.* **19**, 231.

Hawes, R. C. & Skavinski, E. R. (1942). Diffusion micromethod for nitrogen. *Industr. Engng Chem.* (Anal. ed.), **14**, 917.

Hegsted, D. M. (1944). The amino-acid requirements of *Lactobacillus arabinosus*, 17–5. *J. biol. Chem.* **152**, 193.

Hendricks, R. H., Thomas, M. D., Stout, M. & Tolman, B. (1942). Conductrometric determination of ammonia. Application to nitrogen distribution studies. *Industr. Engng Chem.* (Anal. ed.), **14**, 23.

Herter, M. (1940). Sources of error in the colorimetric estimation of nitrogen with Nessler's reagent. *Dtsch. Apoth. Ztg*, **55**, 375 (via *Chem. Abstr.* **35**, 1350^9).

Hevesy, G., Langstrøm-Lang, K., Keston, A. S. & Carsten, O. (1940). Exchange of nitrogen atoms in the leaves of the sunflower. *C.R. trav. Lab. Carlsberg*, Ser. Chim., **23**, 213 (via *Chem. Abstr.* **35**, 1835).

Hier, S. W. & Bergeim, O. (1945). Microbiological determination of free leucine, isoleucine, valine and threonine in dog plasma. *J. biol. Chem.* **161**, 717.

Hier, S. W. & Bergeim, O. (1946). The microbiological determination of certain free amino-acids in human and dog plasma. *J. biol. Chem.* **163**, 129.

Hier, S. W., Graham, C. E., Freides, R. & Klein, D. (1945). The microbiological determination of amino-acids in animal proteins. *J. biol. Chem.* **161**, 705.

Hoagland, R. D. (1944). *Lectures in the Inorganic Nutrition of Plants.* Chronica Botanica Co., Waltham Press.

Hoch, H. (1940). A micro-Kjeldahl apparatus. *Biochem. J.* **34**, 1209.

Hodson, A. Z. (1945). The use of *Neurospora* for the determination of choline and biotin in milk products. *J. biol. Chem.* **157**, 383.

Hoffman, W. S. & Osgood, B. (1940). A photoelectric micro-determination of nitrogenous constituents of blood and urine by direct Nesslerisation. *J. lab. clin. Med.* **25**, 856.

Horowitz, N. H. & Beadle, G. W. (1943). A microbiological method for the determination of choline by use of a mutant of *Neurospora*. *J. biol. Chem.* **150**, 325.

Hutchings, G. H. and Fiske, C. H. (1941). The determination of the purines. *J. biol. Chem.* **140**, 491.

Jacobi, H. P., Baumann, C. A. & Meek, W. J. (1941). The choline content of rats on various choline-free diets. *J. biol. Chem.* **138**, 571.

Johnson, M. J. (1941). Isolation and properties of a pure yeast polypeptidase. *J. biol. Chem.* **137**, 575.

Kalckar, H. M. (1945). Enzymatic micro-analysis of purine compounds. *J. biol. Chem.* **158**, 313.

Kaweran, E. (1941). Estimation of ammonia and urea by a modification of the Conway diffusion method. *Sci. Proc. R. Dublin Soc.* **22**, 405.

Kelley, O. J., Hunter, A. S. & Sterges, A. J. (1946). Determination of nitrogen, phosphorus, potassium, calcium and magnesium. Semi-micro wet-digestion method for large numbers of samples. *Industr. Engng Chem.* (Anal. ed.), **18**, 319.

Kendrick, A. B. & Hanke, M. E. (1940). The effect of iodine and mercury on amino nitrogen values with nitrous acid. *J. biol. Chem.* **132**, 739.

Kerr, S. E. (1940). The determination of purine nucleotides and nucleosides in blood and plasma. *J. biol. Chem.* **132**, 147.

Kerr, S. E. & Seraidarian, K. (1945). The separation of purine nucleosides from free purines and the determination of the purines and ribose in these fractions. *J. biol. Chem.* **159**, 211.

Kieselbach, R. (1944). Microdetermination of nitrates by the Devarda method. *Industr. Engng Chem.* (Anal. ed.), **16**, 746.

Kuiken, K. A., Norman, W. H., Lyman, C. M., Hale, F. & Blotter, L. (1943). The microbiological determination of amino-acids. I. Valine, leucine and isoleucine. *J. biol. Chem.* **151**, 615.

La Cour, L. F. & Drew, R. (1947). Partition-chromatography and living cells. *Nature, Lond.*, **159**, 307.

Lecoq, H. (1940). Dispositif nouveau pour le dosage de l'azote après kjeldahlisation. *Bull. Soc. Chim. biol., Paris*, **22**, 112.

Lewis, J. C. & Olcutt, H. S. (1945). A lactobacillus assay method for *l*-(+)-glutamic acid. *J. biol. Chem.* **157**, 265.

Lintzel, W. & Monasterio, G. (1931). Eine Methode zur Mikrobestimmung des Lecithins im Blute und Plasma. *Biochem. Z.* **241**, 273.

Luecke, R. W. & Pearson, P. B. (1944*a*). The microbiological determination of free choline in plasma and urine. *J. biol. Chem.* **153**, 259.

Luecke, R. W. & Pearson, P. B. (1944*b*). The determination of free choline in animal tissues. *J. biol. Chem.* **155**, 507.

Lugg, J. W. H. & Weller, R. A. (1944). Large scale extraction of protein samples reasonably representative of the whole proteins in the leaves of some plants: the amide, tyrosine, tryptophan, cystine (plus cysteine) and methionine contents of the preparations. *Biochem. J.* **38**, 408.

Lyman, C. M., Kuiken, K. A., Blotter, L. & Hale, F. (1945). The microbiological determination of amino-acids. II. Glutamic acid. *J. biol. Chem.* **157**, 395.

Ma, T. A. & Zuazaga, G. (1942). Micro-Kjeldahl determination of nitrogen. A new indicator and an improved rapid method. *Industr. Engng Chem.* (Anal. ed.), **14**, 280.

MacFadyen, D. A. (1944). Determination of ammonia evolved from α-amino-acids by ninhydrin. *J. biol. Chem.* **153**, 507.

McMahan, J. R. & Snell, E. E. (1944). The microbiological determination of amino acids. I. Valine and arginine. *J. biol. Chem.* **152**, 83.

Mácola, B. A. & Fazio, C. (1941). Determination of the proteins of blood by micro-Kjeldahl and direct Nesslerisation: total proteins, globulins, albumin and fibrinogen. *Analyst*, **66**, 448.

Marenzi, A. D. & Cardini, C. E. (1943). The colorimetric determination of choline. *J. biol. Chem.* **147**, 363.

Markham, R. (1942). A steam distillation apparatus suitable for micro-Kjeldahl analyses. *Biochem. J.* **36**, 790.

Martinek, R. G., Kirch, E. R. & Webster, G. L. (1943). Determination of nicotinic acid. *J. biol. Chem.* **149**, 245.

Mason, M. F. & Rozzell, J. D. (1940). Microdetermination of ammonia. *Proc. Soc. Exp. biol., N.Y.*, **43**, 274.

Melnick, D., Oser, B. L. & Siegel, L. (1941). Chemical determination of nicotinic acid content of flour and bread. *Industr. Engng Chem.* (Anal. ed.), **13**, 879.

Menke, W. (1938*a*). Untersuchungen der einzelnen Zellorgane in Spinatblättern auf Grund präparativ-chemischer Methodik. *Z. Bot.* **32**, 273.

Menke, W. (1938*b*). Untersuchungen über das Protoplasma grüner Pflanzenzellen. I. Isolierung von Chloroplasten aus Spinatblättern. *Hoppe-Seyl. Z.* **257**, 43.

Menke, W. (1940). Untersuchungen über das Protoplasma grüner Pflanzenzellen. II. Der Chlorophyllgehalt der Chloroplasten aus Spinatblättern. *Hoppe-Seyl. Z.* **263**, 100.

Miller, L. & Houghton, J. A. (1945). The micro-Kjeldahl determination of the nitrogen content of amino-acids and proteins. *J. biol. Chem.* **159**, 373.

Modeer, E. (1940). The effect of halogen compounds on Kjeldahl nitrogen digestions. *Univ. Wyoming Publ. Sci.* **7**, 13.

Moore, R. H. (1938). A micro-Kjeldahl method including nitrates. *Bot. Gaz.* **100**, 250.

Neish, A. C. (1939). Studies on chloroplasts. I. Separation of chloroplasts. A study of factors affecting their flocculation and the calculation of the chloroplast content of leaf tissue from chemical analyses. *Biochem. J.* **33**, 293.

Neuberger, A. & Sanger, F. (1942). The nitrogen of the potato. *Biochem. J.* **36**, 662.

Orcutt, F. S. & Wilson, P. W. (1936). Biochemical methods for the study of nitrogen metabolism in plants. *Plant Physiol.* **11**, 718.

Pepkowitz, L. P., Prince, A. L. & Bear, F. E. (1942). A rapid Kjeldahl digestion method using perchloric acid. *Industr. Engng Chem.* (Anal. ed.), **14**, 856.

Pepkowitz, L. P. & Shive, J. W. (1942). Kjeldahl nitrogen determination. A rapid wet-digestion micromethod. *Industr. Engng Chem.* (Anal. ed.), **14**, 914.

Plimmer, R. H. A. (1925). The action of nitrous acid upon amides and other 'amino' compounds. *J. Chem. Soc.* **127**, 2651.

Pope, C. G. & Stevens, M. F. (1939). The determination of amino-nitrogen using a copper method. *Biochem. J.* **33**, 1070.

Prater, A. N., Cowles, E. J. & Straka, R. P. (1942). Determination of ammonia by a diffusion method. *Industr. Engng Chem.* (Anal. ed.), **14**, 703.

Pucher, G. W., Leavenworth, C. S. & Vickery, H. B. (1930). Determination of total nitrogen of plant extracts in presence of nitrates. *Industr. Engng Chem.* (Anal. ed.), **2**, 191.

Pucher, G. W. & Vickery, H. B. (1940). A method for determining glutamine in plant tissues. *Industr. Engng Chem.* (Anal. ed.), **12**, 27.

Rahn, H. (1932). Untersuchungen über den *N*-Stoffwechsel pflanzlicher vegetativer Speicherorgane. *Planta*, **18**, 1.

Ranker, E. R. (1927). Modification of the salicylic-thiosulphate method suitable for determination of total nitrogen in plants, plant solutions and soil extracts. *J. Ass. off. agric. Chem., Wash.*, **10**, 230.

Reifer, I. (1939*a*). Nitrate-nitrogen in plant material. I. Micro-determination of nitrate. *N.Z. J. Sci. Tech.* **20**B, 341.

Reifer, I. (1939*b*). Nitrate-nitrogen in plant material. II. Micro-determination of total nitrogen in presence of nitrate. *N.Z. J. Sci. Tech.* **20**B, 344.

Reifer, I. (1941). Microdetermination of betaine and choline. *N.Z. J. Sci. Tech.* **22**B, 111.

Richards, F. J. & Templeman, W. G. (1936). Physiological studies in plant nutrition. IV. Nitrogen metabolism in relation to nutrient deficiency and age in leaves of barley. *Ann. Bot., Lond.*, **50**, 367.

Rider, B. F. & Mellon, M. G. (1946). Colorimetric determination of nitrites. *Industr. Engng Chem.* (Anal. ed.), **18**, 96.

Riesen, W. H., Schweigert, B. S. & Elvehjem, C. A. (1946). Microbiological determination of methionine in proteins and foodstuffs. *J. biol. Chem.* **165**, 347.

Roberts, M. & James, W. O. (1947). A method for the estimation of total alkaloids in belladonna and stramonium. *Quart. J. Pharm.* **20**, 1.

Roller, E. M. & McKaig, N., Jr. (1939). Some critical studies of the phenoldisulfonic acid method for the determination of nitrates. *Soil Sci.* **47**, 397.

✓ Russell, J. A. (1944). Note on the colorimetric determination of amino-nitrogen. *J. biol. Chem.* **156**, 467.

Ryan, F. J. & Brand, E. (1944). A method for the determination of leucine in protein hydrolysates and in foodstuffs by use of a *Neurospora* mutant. *J. biol. Chem.* **154**, 161.

Schlenker, F. S. (1940). Determination of ammonia, asparagine and glutamine in plant juice. *Plant Physiol.* **15**, 701.

Schlenker, F. S. (1943). A system of analysis for plant tissue by use of plant juice. *Plant Physiol.* **18**, 141.

Schmidt, C. L. A. (1938). *The Chemistry of the Amino-acids and Proteins.* Baltimore: Thomas.

Schmidt, G. & Thannhauser, S. J. (1945). A method for the determination of desoxyribonucleic acid, ribonucleic acid and phosphoproteins in animal tissues. *J. biol. Chem.* **161**, 83.

Schneider, W. C. (1945*a*). Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid, and of pentose nucleic acid. *J. biol. Chem.* **161**, 293.

Schneider, W. C. (1945*b*). Phosphorus compounds in animal tissues. III. A comparison of methods for the estimation of nucleic acid. *J. biol. Chem.* **161**, 747.

Schott, H. F., Rockland, L. B. & Dunn, M. S. (1944). Investigations of amino-acids, peptides and proteins. XVI. A source of error in the manometric ninhydrin method for the analysis of amino-acids and its suppression by the use of hydrazine. *J. biol. Chem.* **154**, 397.

Schweigert, B. S., McIntire, J. M., Elvehjem, C. A. & Strong, F. M. (1944). The direct determination of valine and leucine in fresh animal tissues. *J. biol. Chem.* **155**, 183.

Scott, E. W. & Bambach, K. (1942). Polarographic determination of nitrates in blood and urine. *Industr. Engng Chem.* (Anal. ed.), **14**, 136.

Sessions, A. C. (1930). Method for the determination of inorganic-N in dried plant tissue. *Soil Sci.* **29**, 285.

Sessions, A. C. & Shive, J. W. (1928). A method for the determination of inorganic nitrogen in plant extracts. *Plant Physiol.* **3**, 499.

Shankman, S., Dunn, M. S. & Rubin, L. B. (1943*a*). The analysis of eight amino-acids by a microbiological method. *J. biol. Chem.* **150**, 477.

Shankman, S., Dunn, M. S. & Rubin, L. B. (1943*b*). The microbiological analysis of seven amino acids with *Lactobacillus casei*. *J. biol. Chem.* **151**, 511.

Shinn, M. B. (1941). Colorimetric method for determination of nitrite. *Industr. Engng Chem.* (Anal. ed.), **13**, 33.

Shirkhande, J. G. (1941). Determination of ammoniacal and nitrate nitrogen in decomposed plant material. *Industr. Engng Chem.* (Anal. ed.), **13**, 187.

Sideris, C. P. & Young, H. Y. (1946). Effect of iron on certain nitrogenous fractions of *Ananas comosus* (L.) Merr. *Plant Physiol.* **21**, 75.

Sims, E. A. H. (1945). Microdetermination of glycocyamine and arginine by means of a synthetic ion exchange resin for chromatographic separation. *J. biol. Chem.* **158**, 239.

Sisco, R. C., Cunningham, B. & Kirk, P. L. (1941). Quantitative drop analysis. XIII. The formol titration of amino-nitrogen. *J. biol. Chem.* **139**, 1.

Smith, E. L. (1940). Chlorophyll as the prosthetic group of a protein in the green leaf. *Science*, **91**, 199.

Smith, E. L. (1941). The chlorophyll-protein compound of the green leaf. *J. gen. Physiol.* **24**, 565.

Sobel, A. E., Hirschman, A. & Besman, L. (1945). A convenient micro-titration method for the estimation of amino-acids. *J. biol. Chem.* **161**, 99.

Sobel, A. E., Mayer, A. M. & Gottfried, S. P. (1944). A convenient titrimetric ultramicromethod for the estimation of urea and Kjeldahl nitrogen. *J. biol. Chem.* **156**, 355.

Steward, F. C. (1932). The absorption and accumulation of solutes by living plant cells. II. A technique for the study of respiration and salt absorption in storage tissue under controlled environmental conditions. *Protoplasma*, **15**, 497.

Steward, F. C. & Street, H. E. (1946). The soluble nitrogen fractions of potato tubers: the amides. *Plant Physiol.* **21**, 155.

Steward, F. C. & Street, H. E. (1947). The nitrogenous constituents of plants. *Ann. Rev. Biochem.* **16**, 471.

Stokes, J. L. & Gunness, M. (1945). Microbiological methods for the determination of amino-acids. I. Aspartic acid and serine. *J. biol. Chem.* **157**, 651.

Stokes, J. L., Gunness, M., Dwyer, I. M. & Caswell, M. C. (1945). Microbiological methods for the determination of amino-acids. II. A uniform assay for the ten essential amino-acids. *J. biol. Chem.* **160**, 35.

Strack, E. & Schwaneberg, H. (1937). Über die Bestimmung von Basen aus tierischen Geweben. *Hoppe-Seyl. Z.* **245**, 11.

Street, H. E., Kenyon, A. E. & Watson, G. M. (1946*a*). The nature and distribution of various forms of nitrogen in the potato. *Ann. appl. Biol.* **33**, 1.

Street, H. E., Kenyon, A. E. & Watson, G. M. (1946*b*). The assimilation of ammonium and nitrate nitrogen by detached potato sprouts. *Ann. appl. Biol.* **33**, 369.

Street, H. E., Kenyon, A. E. & Watson, G. M. (1946*c*). The estimation of free choline in plants. *Biochem. J.* **40**, 869.

Stuart, N. W. (1935). The determination of amino nitrogen in plant extracts. *Plant Physiol.* **10**, 135.

Taylor, W. H. & Smith, G. F. (1942). Micro-Kjeldahl nitrogen determination without use of titration procedure. *Industr. Engng Chem.* (Anal. ed.), **14**, 437.

Teiri, A. E. & Shimer, S. R. (1944). A colorimetric determination of nicotinic acid. *J. biol. Chem.* **153**, 307.

Thierfelder, H. & von Cramm, E. (1919). Über glutaminhaltige Polypeptide und zur Frage ihres Vorkommens im Eiweiss. *Hoppe-Seyl. Z.* **105**, 58.

Thornton, M. H. & Broome, F. K. (1942). Determination of choline. A photometric modification of Beattie's method. *Industr. Engng Chem.* (Anal. ed.), **14**, 39.

Umbreit, W. W. & Bond, V. S. (1936). Analysis of plant tissue. Application of a semi-micro-Kjeldahl method. *Industr. Engng Chem.* (Anal. ed.), **8**, 276.

Umbreit, W. W. & Wilson, P. W. (1936). Determination of basic nitrogen. *Industr. Engng Chem.* (Anal. ed.), **8**, 361.

Van Slyke, D. D. (1912). The quantitative determination of aliphatic amino-groups. *J. biol. Chem.* **12**, 275.

Van Slyke, D. D. (1913). The gasometric determination of aliphatic amino-nitrogen in minute quantities. *J. biol. Chem.* **16**, 121.

Van Slyke, D. D. (1915). Note on the micro-method for gasometric determination of aliphatic amino nitrogen. *J. biol. Chem.* **23**, 407.

Van Slyke, D. D. & Dillon, R. T. (1936). Gasometric determination of carboxyl groups in amino-acids. *Proc. Soc. exp. Biol., N.Y.*, **34**, 362.

Van Slyke, D. D. & Dillon, R. T. (1938). Gasometric determination of carboxyl groups in amino acids. *C.R. trav. Lab. Carlsberg*, Ser. Chim., **22**, 480.

Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A. & Hamilton, P. (1941). Gasometric determination of carboxyl groups in free amino-acids. *J. biol. Chem.* **141**, 627.

Van Slyke, D. D., MacFadyen, D. A. & Hamilton, P. (1941). Determination of free acids by titration of the carbon dioxide formed in the reaction with ninhydrin. *J. biol. Chem.* **141**, 671.

Vickery, H. B. & Pucher, G. W. (1929). Determination of nitrate nitrogen in tobacco. *Industr. Engng Chem.* (Anal. ed.), **1**, 121.

Vickery, H. B., Pucher, G. W., Clark, H. E., Chibnall, A. C. & Westall, R. G. (1935). The determination of glutamine in the presence of asparagine. *Biochem. J.* **29**, 2710.

Vickery, H. B., Pucher, G. W. & Leavenworth, C. S. (1935). Determination of ammonia and of amide nitrogen in plant tissues. *Industr. Engng Chem.* (Anal. ed.), **7**, 152.

Vickery, H. B., Pucher, G. W., Schoenheimer, R. & Rittenberg, D. (1940). The assimilation of ammonia nitrogen by the tobacco plant, a preliminary study with isotopic nitrogen. *J. biol. Chem.* **135**, 531.

Vickery, H. B., Pucher, G. W., Wakeman, A. J. & Leavenworth, C. S. (1939). Chemical investigations of the rhubarb plant. *Bull. Conn. agric. Exp. Sta.* no. 424.

Viets, F. G., Jr., Moxon, A. L. & Whitehead, E. I. (1946). Nitrogen metabolism of corn (*Zea mays*) as influenced by ammonium nutrition. *Plant Physiol.* **21**, 271.

Wagner, E. C. (1940). Titration of ammonia in presence of boric acid. In the macro-, semimicro- and micro-Kjeldahl procedures using methyl red indicator and the colour-matching end-point. *Industr. Engng Chem.* (Anal. ed.), **12**, 771.

Waisman, H. A. & Elvehjem, C. A. (1941). Chemical estimation of nicotinic acid and vitamin B_6. *Industr. Engng Chem.* (Anal. ed.), **13**, 221.

Wang, Y. L. & Kodicek, E. (1943). Vitamin methods. VII. A new modification of the β-aminocetophenone method for the estimation of nicotinic acid in urine. *Biochem. J.* **37**, 530.

Went, F. W. (1943). Plant growth under controlled conditions. I. The air-conditioned greenhouses at the California Institute of Technology. *Amer. J. Bot.* **30**, 157.

White, L. M. & Secor, G. E. (1946). Micro-Kjeldahl determination of nitrogen in gramicidin and tryptophan. Comparison of the Gunning-Arnold-Dyer and Friedrich methods. *Industr. Engng Chem.* (Anal. ed.), **18**, 457.

White, R. P. (1943). *A Handbook of Plant Tissue Culture.* Pennsylvania: J. Cattell Press.

Wicks, L. F. (1941). A cheap Nessler's reagent by the use of mercuric oxide. *J. lab. clin. Med.* **27**, 118.

Wicks, L. F. & Firminger, H. I. (1942). Perchloric acid in micro-Kjeldahl digestions. *Industr. Engng Chem.* (Anal. ed.), **14**, 760.

Wildman, S. G. & Bonner, J. (1947). The proteins of green leaves. I. Isolation, enzymatic properties and auxin content of spinach cytoplasmic proteins. *Arch. Biochem.* **14**, 381.

Williams, R. F. (1945). The estimation of nucleic-acid phosphorus in plant material. *Aust. J. exp. Biol. med. Sci.* **23**, 213.

Woiwod, A. J. (1948). Micro estimation of amino-nitrogen and its application to paper partition chromatography. *Nature, Lond.*, **161**, 169.

Wolf, B. (1943). Rapid determination of soluble nutrients in soil and plant extracts by means of a photoelectric colorimeter. *Industr. Engng Chem.* (Anal. ed.), **15**, 248.

✗ Wolf, B. (1944*a*). Rapid photometric determination of total nitrogen phosphorus and potassium in plant material. *Industr. Engng Chem.* (Anal. ed.), **16**, 121.

✓ Wolf, B. (1944*b*). Determination of nitrate, nitrite and ammonium nitrogen. Rapid photometric determination in soil and plant extracts. *Industr. Engng Chem.* (Anal. ed.), **16**, 446.

Wood, J. G. (1945). Nitrogenous constituents of plants. *Ann. Rev. Biochem.* **14**, 665.

Wood, J. G. & Cruikshank, D. H. (1944). The metabolism of starving leaves. V. Changes in amounts of some amino-acids during starvation of grass leaves; and their bearing on the nature of the relationship between proteins and amino-acids. *Aust. J. exp. Biol. med. Sci.* **22**, 111.

Wood, J. G., Cruikshank, D. H. & Kuchel, R. H. (1943). The metabolism of starving leaves. I. Presentation of data; the nature of respiration rate/time curves in air and in nitrogen and the relation to carbohydrates. II. Changes in the amounts of total and chloroplast proteins, chlorophyll, ascorbic acid and soluble nitrogen compounds. III. Changes in malic and citric acid contents and inter-relationships of these with soluble nitrogen compounds. *Aust. J. exp. Biol. med. Sci.* **21**, 37.

Wooley, J. G. & Sebrell, W. H. (1945). Two microbiological methods for the determination of *l*(-)-tryptophane in proteins and other complex substances. *J. biol. Chem.* **157**, 141.

Wurzler, R. J. & Meserve, E. R. (1945). Spectrophotometric determination of small amounts of choline. *J. biol. Chem.* **159**, 395.

Zittle, C. A. & Eldred, N. R. (1944). Determination of *l*-lysine with a specific decarboxylase. *J. biol. Chem.* **156**, 401.

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STUDIES ON THE pH-RELATIONSHIPS OF ROOT GROWTH AND ITS INHIBITION BY 2:4-DICHLOROPHENOXYACETIC ACID AND COUMARIN

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(With 7 figures in the text)

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INTRODUCTION

The role of hydrogen-ion concentration in cell physiology has long been recognized as fundamental, and investigations on the part it plays in both the biochemical and biophysical make-up of the cell have added many basic facts to our store of knowledge of vital processes. It is not surprising, therefore, that many workers should have sought to bring hydrogen-ion concentration to the forefront of factors controlling the activity and growth of the plant cell.

One of the earliest theories on these lines (Strugger, 1932, 1934) was that pH controls growth directly by regulating the degrees of swelling (hydration) of protoplasmic colloids, but this must be dismissed as unlikely in view of subsequent work on the same lines (see J. Bonner, 1934; van Santen, 1938, 1940). A possible effect of pH working, however, via auxin activity, was suggested by the work of Dolk & Thimann (1932), who showed that this activity was highest in an acid medium. This has been subsequently verified for a variety of plant-growth hormones in a range of growth reactions (Marmer, 1937; van Overbeek & Went, 1937; D. M. Bonner, 1938; Noirfalise, 1940). A reasonably close fit of pH-growth curves to the theoretical auxin dissociation curve ($pK = 4{\cdot}75$) was obtained for *Avena* coleoptiles (J. Bonner, 1934), the implications being that auxin is active only in the undissociated form. Similar results were obtained by van Santen (1938, 1940) both for *Avena* coleoptiles and for pea and sunflower roots. More recently, however, Lundegårdh (1946) has claimed that in the growth of root hairs auxin is effective only as an anion, but his experimental data would seem to be insufficient to warrant such a claim.

Thus a number of fundamental points concerning the inter-relationships of pH, auxins and growth still remain obscure. Results for the growth of roots, on which the pH effects should be most easily susceptible of study under normal conditions for growth, are still conflicting (cf. Strugger, 1932; Marmer, 1937; Lane, 1936; van Santen, 1940). In addition, the effects of pH on root-growth inhibitions by growth substances (see Marmer, 1937) needs further study, especially in regard to the action of the recently discovered and highly active molecule, 2:4-dichlorophenoxyacetic acid (2:4-D). Experiments were therefore designed to study these two phenomena. Further, in view of the non-acid nature of the root-growth inhibitor, coumarin (Audus & Quastel, 1947), it was thought desirable to investigate the relationship of its activity to pH.

EXPERIMENTAL DETAILS

At the outset of the work it was realized that the nature and concentration of the buffer solutions used might, to a very large extent, determine the growth rate and might also account, at least partially, for discrepancies in the results of other workers. Thus van Santen (1938), who used phosphate buffers, objected to Michaelis's acetate buffer (used by Strugger) and to McIlvaine's citrate buffer (used by J. Bonner) as being toxic to *Helianthus* after a few hours.

Preliminary experiments were therefore carried out on the effects of buffers themselves on the growth of cress roots. Seeds (20 in a sample) were germinated and grown in test-tubes on the surface of 15 ml. of a liquid culture medium, as in the technique described in a previous paper (Audus & Quastel, 1947). In the first experiments, mixtures of K_2HPO_4 and KH_2PO_4 solutions were employed as buffers, giving a maximum pH range of 4·5–8·8, the final total concentration in the culture medium being 0·015 M. With these concentrations normal growth was obtained, but over the experimental growth period (5–6 days) considerable drifts in the pH were recorded by measurements of initial and final values with a Beckman pH meter using a glass electrode. These shifts were greatest at the alkaline end of the pH range. Typical results for such an experiment for a 7-day growth period are seen in Fig. 1A, where the gross changes in pH are plotted against the initial pH values. The S-shaped curve seen here is a reflexion of the falling-off of buffering capacity at the two extremes of the pH range, the point of inflexion occurring at roughly the theoretical value of 6·8. In addition, however, there is a bodily shift of the whole curve in a direction of increased acidity, due probably to an accumulation of respiratory CO_2 in the medium.

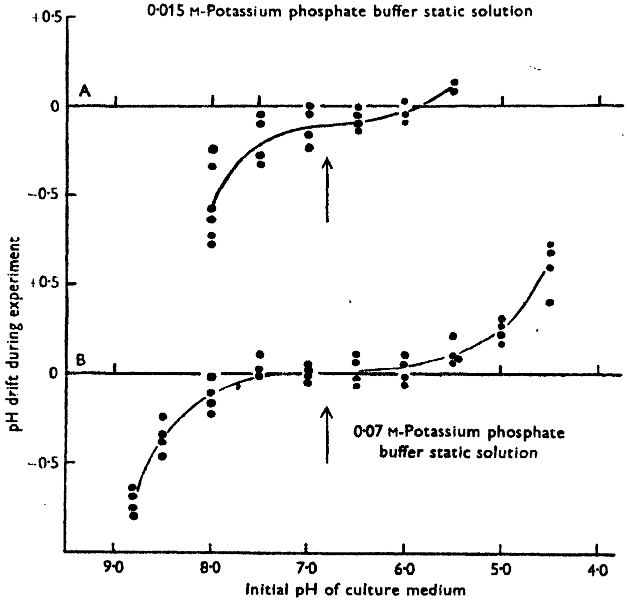

Fig. 1. Graphs showing gross pH-drifts in water-culture solutions of different initial pH values after 7–10 days' growth therein of cress (*Lepidium sativum*) roots.

Subsequently, experiments were carried out with much more concentrated buffer solutions (0·07 M) to see how far this pH drift could be eliminated. These strong solutions, however, considerably reduced the growth rate of the seedlings, especially on the alkaline side (see later results), suggesting a toxic action of the buffer itself. The pH drifts in these solutions (Fig. 1B) over a range of pH of 5·5–8·0 was reduced very considerably in relation to that in the weak buffers over the same range. Equally large drifts were observed, however, in the extreme values of the extended pH range (i.e. from pH 8·0 to 8·8 and pH 4·5 to 5·5).

From these results it was obvious that the employment of weak buffer solutions under these experimental conditions and for these growth periods gave too small a buffering action, even at the point of maximum buffering. In addition, in the static conditions existing in the solutions in the test-tubes, considerable pH gradients probably existed in the close proximity of the root, and the pH as measured was far from representing the true conditions of hydrogen-ion concentration at the root surface. The use of stronger buffers was not favoured, because of their toxic action in high concentrations (further details of this action will be given later).

It was therefore desirable that some technique should be employed in which the roots could be kept bathed in a constant slowly flowing stream of dilute culture medium of a constant pH. In order to do this the apparatus illustrated in Fig. 2 was devised and constructed.

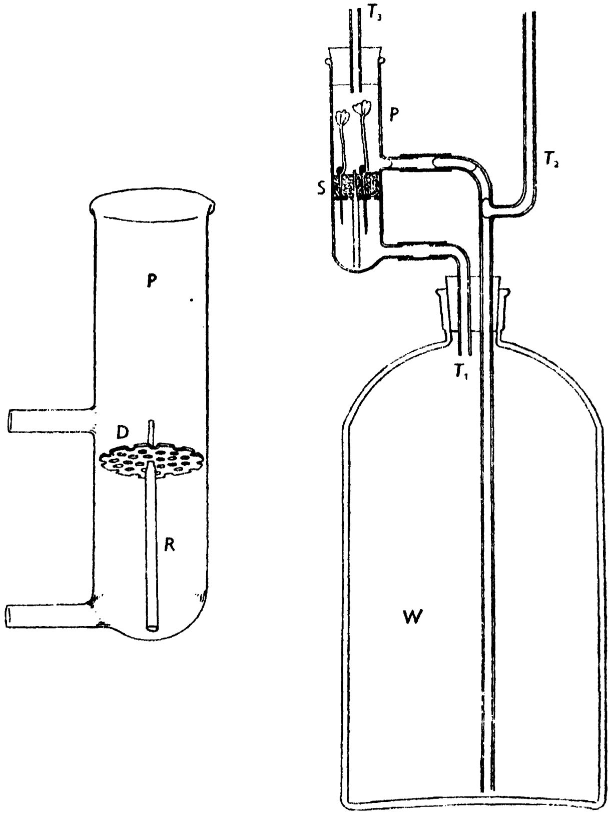

Fig. 2. Apparatus employed for the maintenance of constant pH conditions around the roots of seedlings growing in aerated solutions of optimal nutrient salt concentrations. (For explanation see text.)

The growth vessel consists of a 1 in. × 6 in. Pyrex boiling tube, *P*, fitted with two lateral arms as drawn in the diagram. At the bottom of this tube is placed a support, made up of a tapered glass rod, *R*, and a perforated disk, *D*, of a tough inert plastic material. The other part of the apparatus comprised a 2½ l. Winchester, *W*, which acts as the main reservoir of culture medium of known pH, and connecting tubes, T_1 and T_2. When ready for use the apparatus is assembled as in the figure and the whole filled to about ½ in. above the top of the plastic disk with the culture fluid. A very thin circular disk of glass-wool about 1½ in. in diameter is now pushed down inside the growth vessel, by the aid of a wide-bore glass tube, until it fits neatly on the top of the disk with its edge in contact with the walls of the glass growth vessel to a height of about ¼ in. above the disk. The tapered end of the glass rod naturally projects through this glass-wool disk. A small quantity of coarse (0·5–1·0 mm.) acid-washed sand is now introduced on the top of this glass-wool 'thimble' and the surface washed flat with a small quantity of distilled water from a wash bottle. As will be seen from the diagram, the thickness (about ½ in.) of the sand layer, S, is such that the surface comes just below the upper of the two side arms of the growth vessel. After this procedure the level of the culture solution was usually above that of the sand surface. By careful easing of the rubber connexion away from the top of the tube T_1, the culture fluid could be allowed to escape and the level adjusted to just below that of the sand surface. Seeds were then placed on the surface of this sand and allowed to germinate. The growth vessel was closed by a rubber bung provided with an outlet tube, T_3. The level of insertion of the inlet side-arm of T_2 was arranged to come about ¾–1 in. below the final working level of the culture medium. In this way, a small steady suction applied at T_3 drew in air at the inlet of T_2 causing a constant slow stream of alternating air bubbles and short columns of culture medium to pass upwards and into the growth vessel by the upper side-arm. The rate of air-flow could be varied at will and, for the experiments to be described, was maintained approximately constant at about 100 c.c./hr. The length of the short water columns could be controlled by the distance of the point of insertion of the side-arm of T_2 below the level of the culture fluid. This was so arranged that a constant slow circulation of culture fluid from the reservoir W and through the growth vessel took place at a rate of about 100 c.c./hr. In this way the whole contents of the Winchester would circulate once every 24 hr.

In the majority of experiments the culture solutions used were of the same strength and composition (except for the buffer salts) as those used in the previous work on seedling growth (Audus & Quastel, 1947). KNO_3 was used as a source of nitrogen to a final concentration of 0·1 %. Over a central range (pH 5·5–8·0) different pH values were obtained by appropriate mixtures of the two salts K_2HPO_4 and KH_2PO_4. For an extended pH range normal solutions of Analar HCl and NaOH were added to solutions of the single salts. All pH values were checked with a Beckman pH meter using a glass electrode. Twelve such pieces of apparatus were usually set up, six acting as controls in normal culture medium and six containing a known concentration of growth inhibitor, the same pH range being used in both sets. During the course of the experiment (5–10 days, depending on the species of seedling) daily checks were made of the pH. By careful manipulation, the growth vessel could be removed from the rest of the apparatus without disturbing the culture fluid inside it, thanks to the capillary action of the sand layer. The medium in W could then be checked for pH and any slight drifts over the previous 24 hr. period adjusted by addition of a drop or two of normal HCl or NaOH. With this large reservoir shifts were very small and occurred usually at the extremes of the pH range only (i.e. at pH 9·0 and 4·5).

With this constant-flow technique all the required conditions could therefore be fulfilled, i.e.:

(1) Optimal concentrations of mineral salts in the culture fluid.

(2) Constant pH of the medium bathing the roots, and elimination of gradients.

(3) Adequate aeration of the culture medium and growth vessel.

The air entering the apparatus was drawn through a filter from outside the laboratory to avoid contamination by laboratory gases such as ethylene, which are thought to have such a marked action on growth-substance distribution and its effects in plants (see Borgström, 1939*b*). In all experiments seedlings were grown in light of constant intensity provided by a tungsten filament lamp of 100 W. at a distance of 25 cm. Temperatures were maintained approximately constant at 20° C.

Seedlings were allowed to grow for a suitable time (viz. until the roots at the optimum pH had reached the bottom of the growth vessel). The seedlings were then removed, the roots cut off with a sharp scalpel, surface dried with filter-paper and then the whole sample weighed rapidly to the nearest 0·1 mg. From these figures the total root growth per seedling and the average rate of root growth per seedling over the growing period (i.e. the duration of the experiment minus the average time for the emergence of the radicle) were calculated.

THE EFFECT OF NATURE AND CONCENTRATION OF THE BUFFER SOLUTION

It was mentioned in the last section that the concentration of the buffer had a large effect on the growth of roots grown in test-tubes in a static liquid medium. In order to determine the exact relationship an experiment was carried out with the apparatus described above in which the same buffer mixtures were used in three different concentrations and for a range of pH from 9·0 to 4·5, all other conditions remaining constant. The buffer mixtures were of K_2HPO_4 and KH_2PO_4. The results of this experiment are seen in Fig. 3 A, where root-growth rates are plotted against pH.

In the weakest buffer solution (0·01 M, curve 1) we observe an optimum growth rate in the region of pH 5. The curve is, however, rather flat over a wide pH range but there is a relatively steep fall-away below pH 5 and above 8. The shape of this curve will be discussed later. For the intermediate concentration (0·05 M, curve 2) the optimum is much sharper although it occurs at the same pH and reaches the same absolute level of growth rate. This coincidence of the two curves at and below pH 5 and the much more rapid fall-away of the latter in pH values above 5 provides evidence of a combined action of hydrogen-ion concentration and concentration of phosphate buffer, the precise nature of which is far from clear. The absolute concentration of electrolyte is still low enough to make it unlikely that the concentration effect is acting via the osmotic water-relationships of the cell. In addition, if this were the case, the coincidence of the two optima would be difficult to explain. It seems much more likely that the concentration effect at high pH values is a specific one (e.g. a toxic action of the HPO_4^{--} or PO_4^{---} ions).

Still higher concentrations of buffer (curve 3) show an extension of the inhibition to the full range of pH, and it seems likely that here an osmotic action may be playing a part.

It is interesting to compare these results with those of van Santen. In Fig. 3 A her results for the growth of isolated root cylinders of *Pisum* in 0·004 N-K_2HPO_4-H_3PO_4 buffer mixtures are plotted as crosses, the vertical scale of growth rate being such that the two optima coincide. The agreement between these results and curve 2 is striking. In spite of the fact that her solutions were much weaker than those of curve 2 it is very tempting to suggest that her results also reflect to some degree the complicating effects of buffer concentration brought out so strongly by the results on cress. This would to a large extent invalidate her conclusions concerning the parallelism of growth rate and concentration of undissociated auxin in the root.

The question therefore arises as to how far the fall in growth rate above pH 5 in the weakest buffer may be due to an associated ion-concentration effect, e.g. availability of calcium or iron ions, rather than a pure effect of pH itself. Thus it seemed highly

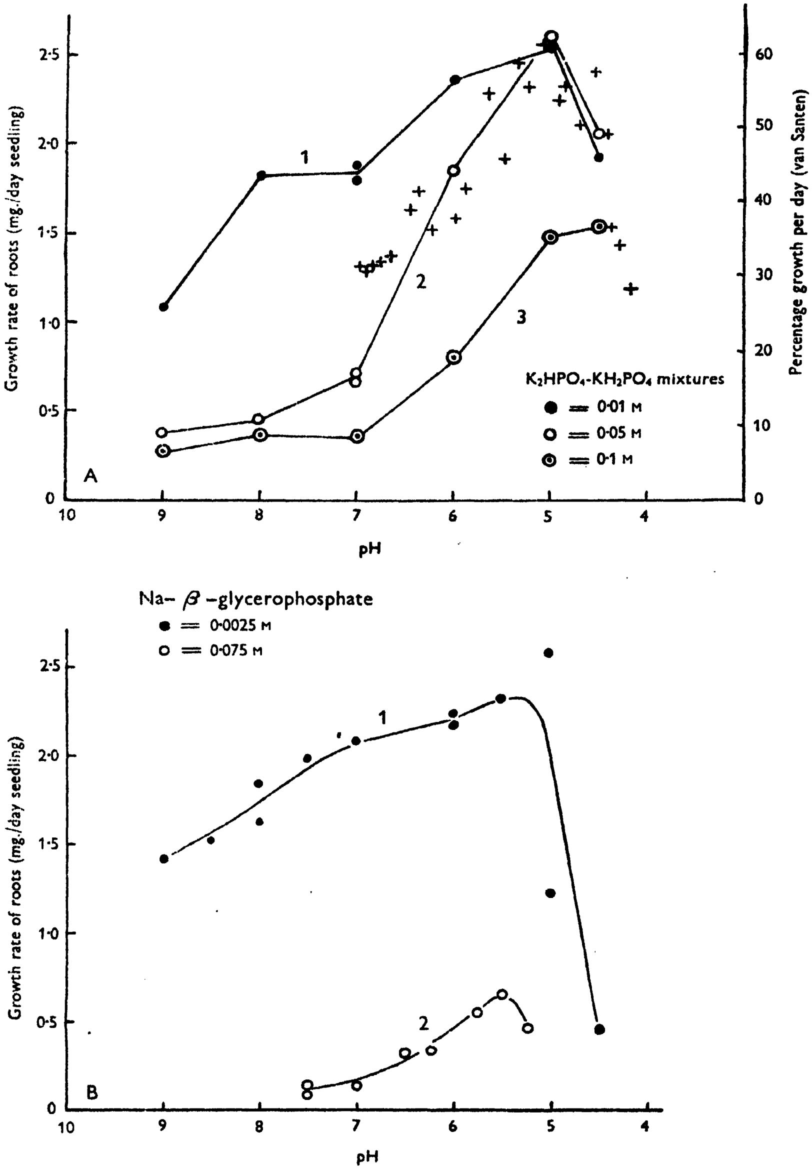

Fig. 3. A, graphs showing the combined effects of pH and potassium phosphate buffer concentrations on the growth of cress (*Lepidium sativum*) roots. The crosses show the results of van Santen (1940) for isolated root cylinders of *Pisum sativum*. B, graphs showing the combined effects of pH and sodium-β-glycerophosphate buffer concentrations on the growth of cress (*Lepidium sativum*) roots.

probable that with the particular culture medium used, where the concentration of calcium ions was determined by the solubility in the culture medium of tribasic calcium phosphate, changes in pH and in relative concentration of the various phosphate ions would bring about a corresponding and possibly a relatively large change in the calcium-ion concentration. A similar state of affairs might exist for iron ions. In order to investigate the possibility of any such change affecting the shape of the pH-growth rate curve, experiments were carried out using sodium-β-glycerophosphate in the combined role of buffer salt and source of phosphate, and using as before HCl and NaOH to adjust the pH values. Calcium was added to this solution in the form of $CaCl_2$. Owing to the relatively high solubility of calcium-β-glycerophosphate the total concentration of calcium in solution should be independent of pH. The concentration of the calcium ion should undoubtedly vary but to nothing like the same extent as in the inorganic phosphate buffers. Two concentrations of the salt were employed, 0·0025 M (i.e. a quarter the concentration of the weakest phosphate buffer) and 0·075 M (i.e. three-quarters the strength of the phosphate buffer).

The results of experiments on these solutions are seen in Fig. 3 B. Curve 1 shows the combined results of experiments with dilute sodium-β-glycerophosphate buffer and comparison with those of Fig. 3 A shows that a form of curve obtained is identical, within the limits of experimental error, with that from weak potassium phosphate buffers. This strengthens the possibility that the curve is a true reflexion of the action of pH *per se*, uncomplicated by any other ionic concentration effect. Curve 2, however, for the strong solution shows very much smaller absolute growth rates but with an optimum pH not significantly different from that of the weak solution. Comparing this with curve 3 in Fig. 3 A we observe a high toxicity of sodium-β-glycerophosphate in relation to that of the potassium phosphates. That this is due to the glycerophosphate and not to the sodium ion is made probable by work on sodium phosphate buffers which gave results comparable with those of the potassium salts. These latter results therefore make it unlikely that the pH-growth rate curve in low concentrations of inorganic phosphate buffer can be explained on a basis of variation in calcium-ion concentration. The question of iron ions has not been investigated. It still leaves unsettled, however, the question as to whether the observed pH-growth curve for low buffer concentrations expresses a true relationship with hydrogen-ion concentration or one which is complicated by correlated changes in the concentrations of nutrient salt ions. The similarity of the two curves 1 in Fig. 3 A, B for two widely different buffer solutions suggests that at this concentration level in cress such effects may very largely have disappeared. More work is needed on this point.

In the experiments which follow potassium phosphate buffer mixtures of 0·01 M concentration have therefore been employed throughout. Tribasic calcium phosphate has been used as a source of calcium ions and the remaining components of the nutrient solutions remaining as in the previous experiments.

THE pH-GROWTH RATE RELATIONSHIP IN CRESS ROOTS

Aggregate results for a number of experiments carried out under identical conditions of light and temperature are collected together in Fig. 4 A (black points). In spite of the considerable degree of scatter it will be seen that the curve exhibits certain definite characteristics which can be specified as follows:

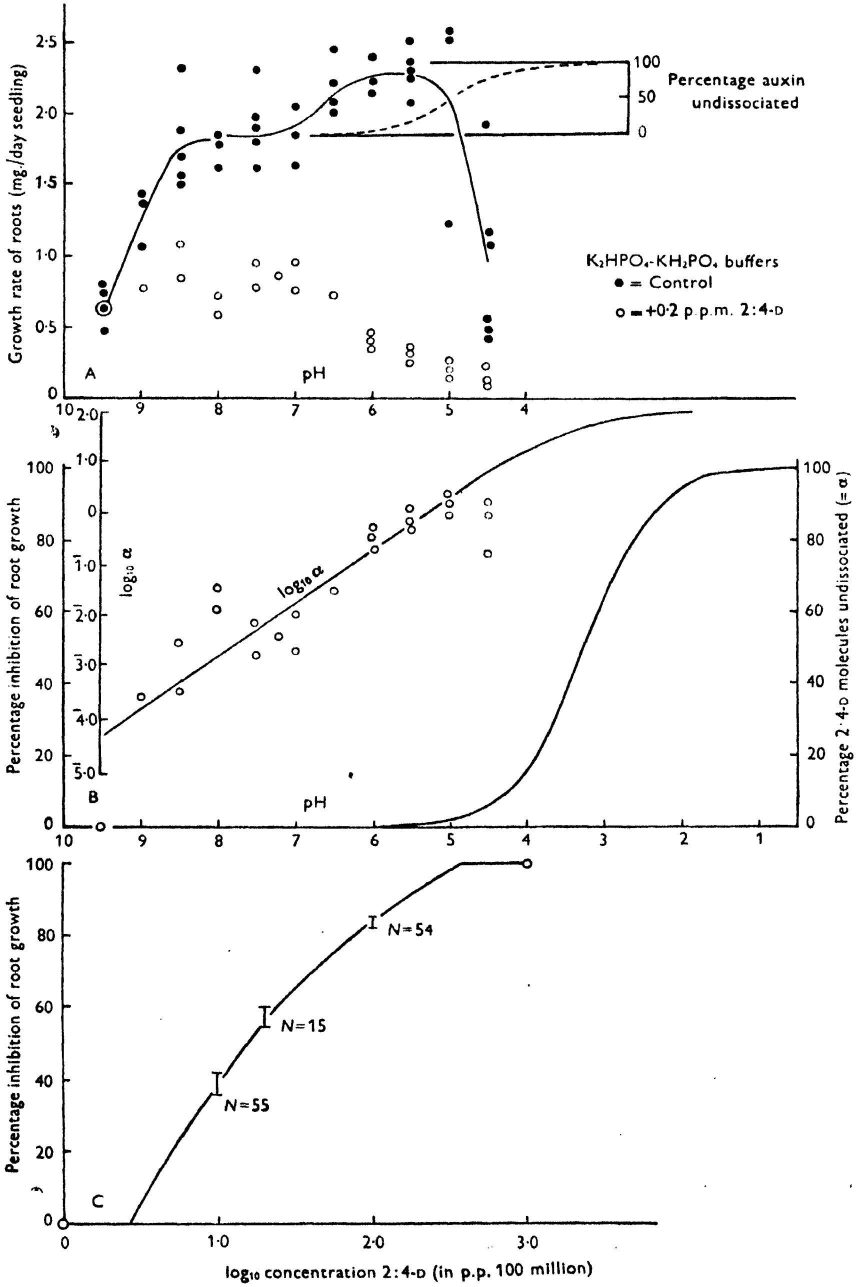

Fig. 4. A, graphs showing the effect of pH on root growth of cress (*Lepidium sativum*) both with and without the addition of 0·2 p.p.m. 2:4-D. B, graphs showing the relationship to pH of the percentage root-growth inhibition by and dissociation characteristics of 2:4-D. C, graph showing the relationship of root-growth inhibition to 2:4-D concentration at a constant pH (6·8).

(1) At the extreme ends of the pH range investigated (i.e. above pH 8·5 and below 5) there is a rapid decrease in the growth rate as we proceed into more alkaline or more acid solutions respectively.

(2) Over the central pH range (i.e. pH 8·5–5·0) pH effects are not so marked, but significantly higher rates obtain over the range of pH 5·0–6·5 than over the range pH 7·0–8·5.

There is, therefore, no indication of a twin-optimum curve as claimed by Strugger (1932). On the other hand, results show very broad resemblences to those of van Santen (1940), although there are differences in detail. In Fig. 4A, using the same value of pK for auxin as van Santen (i.e. 5·0), a dissociation curve has been drawn by the side of the observed values of growth rate over this central range of pH. This dotted curve shows the percentage of undissociated auxin molecules in solution plotted against the same pH scale.

Before making any comparisons of these two curves it would be as well to recall the differences that exist between the reactions of coleoptile and root to auxins. In the experiments of J. Bonner and van Santen it was tacitly assumed that, over the range of concentrations of undissociated auxin molecules existing in the cell, the growth rate was strictly proportional to the concentration. This assumption is a necessary basis for the exact fitting of their theoretical and observed curves. In the case of the coleoptile they were probably justified, since it is well known that a proportionality exists in low auxin concentrations between total concentration and growth rate (see Went & Thimann, 1937, pp. 38–41). In the root, however, there is still uncertainty regarding the corresponding relationship. At a certain concentration (within the range 0·1 and 0·01 p.p.m. in cress) and at pH 6·8 a definite inhibition of growth sets in and increases progressively with, and roughly proportional to, the logarithm of the concentration. Below this concentration a number of workers claim to have shown small but significant stimulations of growth rate with external heteroauxin concentrations of the order of 1 part in 10^{10} (Amlong, 1936; Fiedler, 1936; Thimann, 1936; Geiger-Huber & Burlet, 1936; Grace, 1937; Macht & Grumbein, 1937; Weiler, 1938; Borgström, 1939*a*). A further complication is introduced by the fact that the pH of the cell sap is probably not the same as that of the surrounding medium, although considerable adjustments of internal pH may take place in response to external conditions (see later, p. 107 and also J. Bonner, 1934; van Santen, 1940). However it would seem that the observed results in Fig. 4A can be plausibly explained along the following lines.

Over the pH range 7·0–8·5 growth rate is relatively constant and may correspond to a state in which the auxin present in the root is almost completely dissociated and therefore without effect on the growth rate. Above pH 8·5, serious disturbances in the electrical properties of the protoplasmic colloids may be the cause of the rapid decrease in the growth rate. At pH 7·0, however, growth starts to increase with increasing acidity. At this point the proportion of undissociated molecules reaches 1%. At pH 5·5, i.e. at the optimum pH for root growth, it has reached approximately 25%. It seems likely that this increase in the growth rate is closely linked with the corresponding increase in the concentration of undissociated auxin molecules, a possibility according well with the aforementioned claims of root-growth stimulation in very low auxin concentrations. Below pH 5·0 the growth rate again starts to decline and by pH 4·5 has reached a value about 30% of the optimum. This agrees closely with the results of van Santen (1940). It might be suggested that at this point (pH 5·0) the concentration of undissociated auxin molecules reaches a value where supraoptimal action (Boysen-Jensen, 1936) becomes apparent and we enter on the inhibiting range of concentration. It has been shown, however (unpublished work by the author), that for a 70% growth inhibition of cress roots at pH 6·8 we require a total concentration of heteroauxin of approximately 0·5 p.p.m. corresponding to a concentration of undissociates molecules of approximately 0·005 p.p.m. Assuming that auxin possesses the same order of root-growth inhibiting activity, then it can be shown that the observed inhibitions at pH 4·5 might be evoked by the normal auxin content of the root (*c.* 0·01 p.p.m.; cf. Thimann, 1934). In view of the fact, however, that this pH probably does not represent that of the cell sap (see p. 107) which is probably much nearer neutrality, this cannot be the whole story, and other unrelated pH effects on cell physiology must be determining growth rates, as above pH 8·5 (external). It is obvious that in the present state of our knowledge attempts at a precise fitting of observed root-growth rates with theoretical curves for auxin dissociation, as carried out by van Santen (1940), are a waste of time.

pH AND 2:4-DICHLOROPHENOXYACETIC ACID INHIBITION OF ROOT GROWTH

Experiments on the effects of pH on root-growth inhibition by 2:4-D were carried out with twelve sets of thirty seedlings each, six sets in normal culture solutions over a range of pH values and six in an exactly similar range of solutions but with the addition of 0·2 p.p.m. 2:4-D. The whole series of experiments were carried out under identical conditions and are thus strictly comparable. The results of all such experiments have been lumped and plotted on the same graph in Fig. 4A. The control values are somewhat more numerous than the inhibited, since a number of additional points have been included from other experiments on pH effects alone. The values for the inhibited growth rate show considerable variations, but the main trend of the graph is a progressive increase in inhibition as one proceeds from the alkaline to the acid end of the pH range. This is seen more clearly in Fig. 4B. Using the smooth control curve of Fig. 4A as a basis of comparison, the percentage inhibition has been calculated for each observed value of the growth rate in 2:4-D and these values (circles) have been plotted against pH. It will be seen that there is a roughly linear increase of inhibition with decreasing pH.

This agrees in principle with the findings of previous workers that increasing acidity is correlated with an increase in the activity of the growth substance. How far, however, can the degree of this inhibition be correlated with the dissociation of the 2:4-D molecule in solution? Dissociation constants for this acid have recently been determined in the laboratories of the Imperial Chemical Industries, Ltd., Blackley, Manchester (data supplied by Dr W. A. Sexton). At a dilution of 0·0005 M (i.e. 110 p.p.m.) the pK of this acid is 3·28, and from this value figures for the percentage dissociation have been determined for a range of pH values. The smooth curve to the right of Fig. 4B shows the relationship to pH of the percentage of the total 2:4-D molecules existing in the undissociated state. It is obvious that no simple correlation exists between this concentration of undissociated molecules and the percentage growth inhibition. It is true that the concentrations used for growth inhibition were much smaller than those used in the determination of the pK value (i.e. 0·2 p.p.m. as compared with 110 p.p.m.). Corrections for the change in pK with increased dilution would, however, reduce only by a relatively small amount the large discrepancy in the fitting of these two sets of data.

It is, however, possible that the results can be explained on a basis of 2:4-D dissociation and the restriction of inhibitory activity to the undissociated molecule. Thus the sensitivity of the root to 2:4-D might be such that the inhibitions are brought about by the extremely low concentrations of undissociated molecules in the pH range of the experiments. It has been found that at constant pH and over its inhibiting range of concentration the inhibition of root growth by 2:4-D is almost directly proportional to the logarithm of the inhibiting concentration, a similar relationship between growth-substance action and concentration being observed by van Overbeek & Went (1937) in the pea test. This is shown in Fig. 4C, where a large number of growth experiments are collected together (for description of methods used see Audus & Quastel, 1947). The lengths of the vertical black lines in this graph represent twice the standard error of the mean of a number of measurements of root-growth inhibition and are plotted on the mean itself as a mid-point. The relevant numbers of observations are marked by the side of each line. It can be shown from the dissociation curve that, over the pH range 9·5–6·5, the relationship between $\log_{10}$ (concentration of undissociated 2:4-D molecules) and pH is also, to all intents and purposes, linear. Presupposing, therefore, an appropriately high inhibitory activity of undissociated 2:4-D molecules, might we not have here a similar direct correlation of growth inhibition with $\log_{10}$ concentration?

To check this possibility, a curve of $\log_{10}$ (concentration of undissociated 2:4-D) $= \log_{10}\alpha$ has been drawn against pH in Fig. 4B to fit as closely as possible to the observed points for growth inhibition. The adjusted scale of $\log_{10}\alpha$ is drawn to the left of the graph. The correlation here observed might be taken as proof that the above explanation of the pH-growth-inhibition relationships is the correct one, until the numerical relationships are observed in more detail. Thus it will be seen from Fig. 4C that for an increase in inhibition from 40 to 80% at constant external pH (i.e. at constant percentage dissociation) an approximately tenfold increase in the concentration of inhibitor is required. From the results in Fig. 4B, however, such an increase in percentage inhibition requires a change of $\log_{10}\alpha$ from $\bar{4}\cdot 7$ to $\bar{1}\cdot 5$ $(=2\cdot 75)$, i.e. an approximately 560-fold increase in α, the concentration of undissociated 2:4-D molecules. This discrepancy in concentration range can be bridged only if we assume that, as a result of cell-buffering action, the effective pH range at the site of action of the inhibitor is much smaller than that in the external solution. A pH range of 7·3–5·5, corresponding to the external pH range of 9·5–4·5, would give a change in $\log_{10}\alpha$ of one unit, i.e. the necessary tenfold increase in the concentration of undissociated inhibitor. We might agree with Lundegårdh (1946) that growth-substance action takes place at the external surface of the cell protoplast and it would then be here, i.e. at the cell-wall-protoplasm interface that this reduced pH range would occur. From the cytological and meristematic effects of such growth substances (see Thompson, 1945) it is more likely that the site of action is in the protoplast. The work of Osterhout (1925) and Osterhout & Dorcas (1925) makes it probable that only undissociated molecules penetrate the cell membrane and it is held that at equilibrium external and internal concentrations of these molecules are equal, the pH of the cell sap remaining unaffected by external changes in hydrogen-ion concentration. Here again we have only to assume the intermediate and partial buffering action of the cell wall to get the appropriate concentration range of undissociated inhibitor in the cell sap, since such concentrations would be equal to those immediately outside in the cell wall. Albaum, Kaiser & Nestler (1937), however, claim that for heteroauxin and *Nitella* cells at equilibrium the total (dissociated plus undissociated) concentrations are the same in the cell sap and in the external medium. J. Bonner (1934) also showed in the *Avena* coleoptile an induced pH range of 4·7–6·9 in the cell sap for a corresponding range of pH in the external medium of 2·5–7·9. Indirect evidence of similar pH changes are brought forward by van Santen (1940). Such a partial pH shift in the root-cell protoplasm, coupled with equality of internal and external concentrations of total inhibitor, would also explain the observed facts. The final decision on these points can be taken only when adequate and conclusive data are obtained concerning the equilibrium conditions of pH and growth-substance concentration at the site of its action in the plant root.

A few additional experiments have been carried out on other plant species, e.g. radish, garden pea and maize, with essentially similar results. Fig. 5 shows the results obtained in an experiment on radish, where exactly similar pH-growth-inhibition relationships are exhibited.

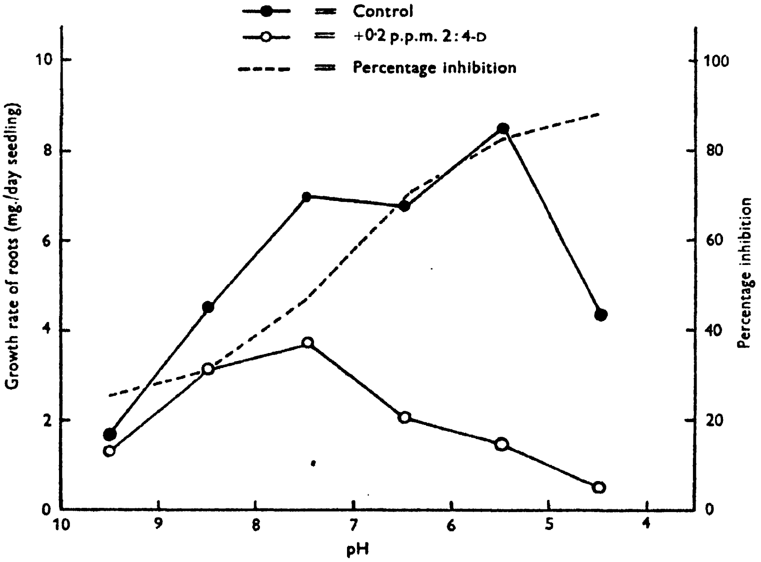

Fig. 5. Graphs showing the effect of pH on the root growth of radish (*Raphanus sativus*) and its inhibition by 0·2 p.p.m. 2:4-D.

Results with coumarin

(1) *Root-growth inhibition*

Similar experiments have been carried out using a concentration range of 10–15 p.p.m. of coumarin for root-growth inhibitions. Cress, carrot, pea and radish were all studied and all gave essentially similar results. Fig. 6 shows a typical result for cress roots. It will be seen that, except for the very low value at pH 9·5, there is no significant change with pH of the degree of coumarin inhibition. This low value at pH 9·5 is not significant since, at the extremes of the pH range, the small growth rates with their relatively large experimental errors give much wider fluctuations in the calculated percentage inhibition.

If we were to assume that growth-substance inhibitions involved precisely the same cell mechanism as coumarin inhibition, then these findings would constitute fairly convincing proof of the dissociation hypothesis of auxin activity. Although there are a number of facts which demonstrate a close similarity of some of the physiological responses of the plant to auxins and to coumarin (Audus & Quastel, 1947; Audus, 1948), yet there are also marked differences (see Veldstra & Havinga, 1943; Larsen, 1947), and many more data are needed before such an assumption is justified.

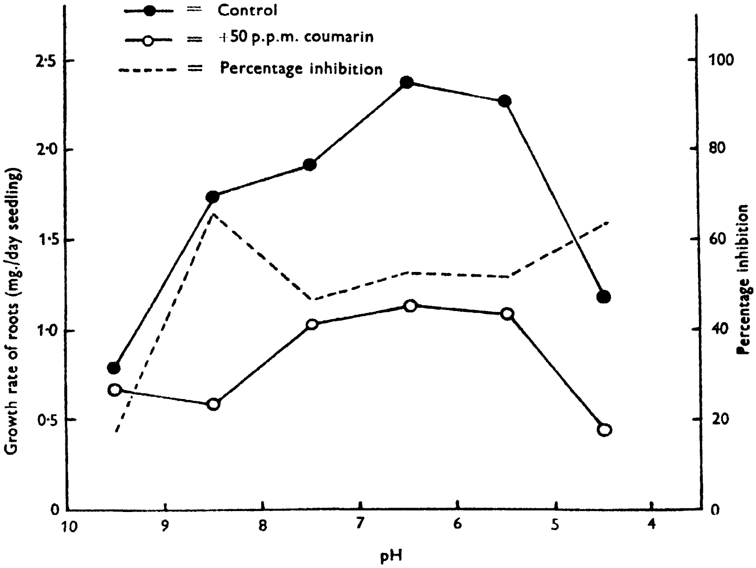

Fig. 6. Graphs showing the effect of pH on root-growth inhibition of cress (*Lepidium sativum*) by 50 p.p.m. coumarin.

(2) *Stimulation of lateral root production*

One of the parallelisms of the actions of auxin and coumarin is brought out strongly in experiments on pH. This is a stimulation of the activity of lateral root meristems resulting in the development of numerous lateral roots. The effect is well known from the literature on plant growth substances. This stimulated meristematic activity results in a considerable swelling of the root, and may eventually cause a sloughing off of the whole cortex of the region exposed to the inhibitor, thereby exposing the numerous lateral meristems as rows of small crowded tooth-like points above the protoxylem. These meristems never grow out if the concentration of the growth substance in the surrounding medium is maintained at the level for longitudinal growth inhibition. On removal of the inhibitor, however, growth recommences and remarkable root-fasciation phenomena thereby result (Audus, 1948). In the case of coumarin a similar but much smaller stimulation of lateral meristems is observed. Here one can obtain considerable stimulation of lateral root meristem activity without complete inhibition of longitudinal growth, with the result that these lateral roots grow out, although they remain stunted and somewhat distorted in shape. Such a phenomenon occurred in all the experiments on coumarin and pH, but here a further complication appeared in the shape of a stimulation of lateral root meristems in the acid culture medium in the absence of coumarin (cf. Marmer, 1937). The extent of this stimulation varied somewhat from species to species, being very high in carrot and low in radish. These results are brought out in Fig. 7, where the density of lateral roots (i.e. number per centimetre of main tap root) is plotted against pH of the external culture medium. An explanation of this phenomenon which immediately springs to mind is that here we have further evidence of the inverse correlation of auxin activity (lateral meristem stimulation) with its dissociation.

In the presence of coumarin, however, there seems to be a general overall stimulation of this meristematic activity over the whole range of pH, although in cress the maximum stimulation still tends to occur at the acid end of the range. These results, which are brought together in the graphs of Fig. 7, are of too scanty and non-uniform a nature to allow of any precise generalizations but they would indicate, however, that in the case of cress at least, pH is affecting lateral root meristem activity quite apart from any direct action on the stimulating molecule. This greater action of coumarin in acid solutions, which cannot be explained by electrolytic dissociation phenomena as in the case of growth substances, may be explained by a direct action of hydrogen ions on the sensitivity of the

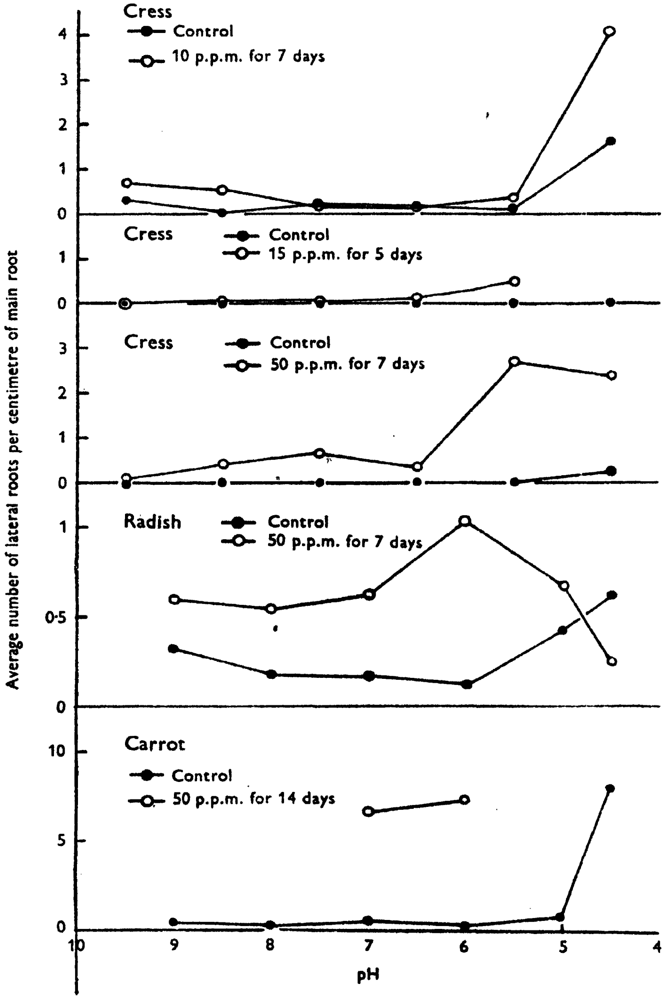

Fig. 7. Graphs showing the effect of pH on lateral root production and its stimulation by various concentrations of coumarin in cress (*Lepidium sativum*), radish (*Raphanus sativus*) and carrot (*Daucus carota*).

lateral meristems, or possibly by a synergistic action of coumarin augmenting the response of the meristem to the active (undissociated) auxin already present.

DISCUSSION

The foregoing results have demonstrated that in work on the pH-growth relationships of plant roots great care must be exercised in the interpretation of results, since the nature and concentration of the particular buffer employed may produce large distortions of the pH curves obtained. Such distortions are presumably due to large associated ionic changes, e.g. high toxic concentrations of HPO_4^{--} or PO_4^{---} ions or vanishing low concentrations of iron ions in alkaline solutions. Further complications arise in that weak buffer solutions are ineffective, the root, presumably by ionic interchange and by the production of respiratory CO_2, rapidly shifting the pH towards a value somewhat on the acid side of neutrality. This, and the considerable pH gradients that must exist in the neighbourhood of roots growing under static conditions of the bathing medium, must account for discrepancies obtained in much of the previous work. By use of dilute culture media, and the mechanical buffering device described, constant conditions could be maintained at the surface of the root, and the growth conditions thereby obtained approximate very closely to the optimum. It seems most probable, therefore, that the relatively flat-topped curve obtained for root growth under these conditions reflects the true pH relationship with minimum complications arising from other variables correlated with the pH. This curve shows no evidence of a double optimum, but the considerable scatter of individual readings shows how such bimodal curves might arise by a biased interpretation of too scanty data. This could easily account for the results of Strugger (1932, 1934).

The finding that the growth optimum occurs on the acid side of neutrality falls in line with previous work on pH-growth relationships in a variety of plant organs. Attempts, however, at a precise interpretation of the curve obtained depend on the assumption that the pH of the cell sap reflects to a considerable extent the pH of the surrounding medium. In spite of conflicting evidence from the literature, some of the facts presented demonstrate that such an adjustment of internal pH is possible, as an explanation of the variation with pH of 2:4-D inhibition of root growth and of the activity of lateral root meristems. The full extent of this pH shift (the claim of van Santen for equality of external and internal pH is certainly not justified) is not known with any certainty and so a precise interpretation of pH-growth curves in terms of the concentration of active (undissociated) auxin molecules would be premature.

The dependence of the growth-inhibitory activity of 2:4-D on pH also agrees in principle with the findings of other workers and observed values of the percentage inhibition of root growth can be explained on the dissociation hypothesis providing the following two assumptions are made:

(1) That root inhibition is proportional to the logarithm of the concentration of undissociated 2:4-D molecules, this assumption according well with findings in experiments at constant pH.

(2) That only a partial adjustment of pH takes place at the site of inhibitor action in response to various pH values in the surrounding medium.

What is clear, however, from these results is that if growth substances are active, both as growth stimulators in plants (in the coleoptile) and as growth inhibitors (in the root), only when in the undissociated form, then the activity of 2:4-D when corrected for this dissociation, is far in advance of that of heteroauxin. Thus 70% inhibition of root growth of cress at pH 6·8 requires 0·5 p.p.m. heteroauxin (9% of the molecules undissociated) and 0·4 p.p.m. of 2:4-D (0·032% of the molecules undissociated). This gives a relative activity of 2:4-D/g. mol. of undissociated acid of approximately 450 times that of heteroauxin. This is in strong contrast with the results of D. M. Bonner (1938), who claimed to have shown that for a range of growth substances differences in activity in the *Avena* coleoptile test were explicable entirely in terms of differences in degrees of dissociation, the activities reckoned in terms of free acid being identical. The above large discrepancy in this inverse correlation between dissociation and growth activity of the growth substances disproves the validity of this generalization and shows that properties other than the dissociation of the —COOH group play a part in determining this physiological activity. This is well illustrated by the work of Veldstra (1944), who showed that a high (lipophilic) surface activity of the ring system of the growth-substance molecule is correlated with high growth promoting activity (pea test). It is presumed that the 'turgescent' action on the complex films of orientated phosphatide ions constituting the protoplasmic membrane is responsible for their growth-regulatory activity, which can therefore be referred ultimately to permeability regulation. Whatever the merits or failings of this particular theory, it is clear that the high root-growth inhibitory activity of 2:4-D can be similarly correlated with high surface activity of its ring system with the two substituted halogen atoms (see Veldstra, 1946). The need for further work along these lines is clear.

The lack of any significant effect of pH on the growth-inhibiting action of coumarin is but rather remote evidence in support of the dissociation hypothesis of growth-substance activity. The parallelism that has been observed in some of the physiological actions of growth substances and coumarin is no proof that their root-growth inhibitions involve the same system. Further research on the similarities and differences of growth substance and coumarin action may well result in the confirmation or otherwise of this dissociation hypothesis.

SUMMARY

Results of experiments on the growth of cress (*Lepidium sativum*) seedlings in water-culture solutions of various concentrations and constitutions with a range of pH values (9·5–4·5) have shown the necessity for exercising great care in the interpretation of pH-growth curves. The toxicity of particular ions and large pH drifts and gradients in the neighbourhood of the roots in weak static buffers may give rise to large distortions in the observed pH-root-growth curves. This, in conjunction with the large biological variations usually obtained under constant environmental conditions in such experiments, may account for the discrepancies in the results of previous workers.

By using a constant flow of dilute culture medium maintained at a constant pH, a relatively flat-topped curve was obtained for growth rate plotted against external pH. Optimum growth was at pH 5·5. It was shown that the form of this curve can be partially explained, in terms of varying auxin activity, assuming that only the undissociated auxin molecule (pK = 5) is active, both as a growth promoter and as a growth inhibitor. The stimulation of the activity of lateral root meristems in acid solutions could also be explained on this basis.

The results of experiments on cress, radish, garden pea and maize seedlings show increasing root-growth inhibition by 2:4-D with decreasing pH over the range 9·5–4·5.

These results may also be explained in terms of 2:4-D dissociation (pK = 3·28), if, as is forecast by experiments at constant pH (6·8), the inhibition is proportional to the logarithm of the concentration of active (undissociated) molecules. 2:4-D, however, forms a glaring exception to the generalization that growth substances show equal activities when these latter are expressed in terms of 'free acid', being approximately 450 times more active as a growth inhibitor than heteroauxin on this basis.

The root-growth inhibiting action of coumarin in cress, radish, carrot and garden pea shows no such variation with pH. There was, however, some indication that its stimulation of the activity of lateral root meristems, which occurs over the whole pH range, was greatest at the acid end.

REFERENCES

Albaum, H. G., Kaiser, S. & Nestler, H. A. (1937). The relation of hydrogen-ion concentration to the penetration of 3-indole acetic acid into *Nitella* cells. *Amer. J. Bot.* **24**, 513.

Amlong, H. U. (1936). Der Einfluss des Wuchsstoffes auf die Wanddehnbarkeit der *Vicia-Faba*-Wurzel. *Ber. dtsch. bot. Ges.* **54**, 271.

Audus, L. J. (1948). Studies on the phytostatic action of 2:4-dichlorophenoxyacetic acid and coumarin. The reversibility of root growth inhibition. *New Phytol.* **47**, 196.

Audus, L. J. & Quastel, J. H. (1947). Coumarin as a selective phytocidal agent. *Nature, Lond.*, **159**, 320.

Bonner, D. M. (1938). Relation of environment and of the physical properties of synthetic growth substances to the growth reaction. *Bot. Gaz.* **100**, 200.

Bonner, J. (1934). The relation of hydrogen ions to the growth rate of the *Avena* coleoptile. *Protoplasma*, **21**, 406.

Borgström, G. (1939*a*). Influence of growth-promoting chemicals on roots of *Allium*. I. Effective concentration ranges of synthetic growth substances and vitamins. *Bot. Notiser*, p. 207.

Borgström, G. (1939*b*). Theoretical suggestions regarding the ethylene responses of plants and observations on the influence of apple-emanations. *K. fysiogr. Sällsk. Lund Förh.* **9**, (12), 1.

Boysen-Jensen, P. (1936). Über die Verteilung des Wuchsstoffes in Keimstengeln und Wurzeln während der phototropischen und geotropischen Krümmung. *Kgl. danske vidensk. Selsk. Biol. Med.* **13**, 1.

Dolk, H. E. & Thimann, K. V. (1932). Studies on the growth hormones of plants. I. *Proc. Nat. Acad. Sci.* **18**, 30.

Fiedler, H. (1936). Entwicklungs- und reiz-physiologische Untersuchungen an Kulturen isolierter Wurzelspitzen. *Z. Bot.* **30**, 385.

Geiger-Huber, M. & Burlet, E. (1936). Über den hormonalen Einfluss der β-Indole-essigsäure auf das Wachstum isolierter Wurzeln in keimfreier Organkultur. *Jb. wiss. Bot.* **84**, 233.

Grace, N. H. (1937). Physiological curve of response to phytohormones by seeds, growing plants, cuttings and lower plant forms. *Canad. J. Res.* Sect. C, **15**, 538.

Lane, R. H. (1936). The inhibition of roots by growth hormones. *Amer. J. Bot.* **23**, 532.

Larsen, P. (1947). *Avena* curvatures produced by mixtures of growth promoting and growth retarding substances. *Amer. J. Bot.* **34** (7), 349.

Lundegårdh, H. (1946). The growth of root hairs. *Ark. Bot.* **33** A, no. 5, 1.

Macht, D. I. & Grumbein, M. L. (1937). Influence of indole-acetic, indole-butyric and naphthalene acetic acids on roots of *Lupins albus* seedlings. *Amer. J. Bot.* **24**, 457.

Marmer, D. R. (1937). Growth of wheat seedlings in solutions containing chemical growth substances. *Amer. J. Bot.* **24**, 139.

Noirfalise, A. (1940). Recherches sur le développement des racines de *Vicia faba* traitées à l'héteroauxine. *La Cellule*, **48**, 309.

Osterhout, W. J. V. (1925). Is living protoplasm permeable to ions? *J. Gen. Physiol.* **8** (1), 131.

Osterhout, W. J. V. & Dorcas, M. J. (1925). The penetration of CO_2 into living protoplasm. *J. Gen. Physiol.* **9** (2), 255.

Overbeek, J. van & Went, F. W. (1937). Mechanism and quantitative application of the pea test. *Bot. Gaz.* **99** (1), 22.

Santen, A. M. A. van (1938). Influence of hydrogen-ion concentrations on the growth rate of the *Avena* coleoptile. *Kon. Akad. Wetensch. Amsterdam: Proc. Sec. Sci.* **41**, 513.

Santen, A. M. A. van (1940). *Groei, groeistof en* pH. Dissertation, University of Utrecht.

Strugger, S. (1932). Die Beeinflussung des Wachstums und des Geotropismus durch die Wasserstoffionen. *Ber. dtsch. bot. Ges.* **50**, 77.

Strugger, S. (1934). Beiträge zur Physiologie des Wachstums. I. Zur protoplasmaphysiologischen Kausalanalyse des Streckungswachstums. *J. wiss. Bot.* **79**, 406.

THIMANN, K. V. (1934). Studies on the growth hormone of plants. VI. The distribution of growth substances in plant tissues. *J. Gen. Physiol.* **18**, 23.

THIMANN, K. V. (1936). Auxins and the growth of roots. *Amer. J. Bot.* **23**, 561.

THOMPSON, B. F. (1945). Tissue responses to physiologically active substances. *Bot. Rev.* **11**, 593.

VELDSTRA, H. (1944). Researches on plant growth substances. IV. Relation between chemical structure and physiological activity. *Enzymologia*, **11**, 97, 137.

VELDSTRA, H. (1946). Enkelen grepen uit chemie en physiologie van Groei- en Remstoffen. *Landbouwk. Tijdschr.* **701/702**, 483.

VELDSTRA, H. & HAVINGA, E. (1943). Untersuchungen über pflanzliche Wuchsstoffe. VII. Über Struktur und Wirkungsmechanismus der pflanzlichen Wuchs- und Hemmstoffe. *Rec. Trav. chim. Pays-Bas*, **62** (12), 841.

WEILER, F. (1938). Das Verhalten der Wurzeln unter der Einwirkung von Wuchsstoffen der *Avena*- und der *Zea*-Koleoptilspitzen. *Bull. Akad. Pol. Sci. et Lettr. Classe Sci.* Math. Nat. Ser. B.

WENT, F. W. & THIMANN, K. V. (1937). *Phytohormones*, pp. 38–41. London: Macmillan and Co.

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THE EFFECT OF PICKING ON THE FLOWERING OF BLUEBELL, *SCILLA NON-SCRIPTA*

BY T. R. PEACE AND J. S. L. GILMOUR

Independently both the authors of this paper, interested in repeated statements that the picking of bluebells lessened their flowering capacity in future years, decided to start experiments to test this effect. The first author's plots were situated in Bagley Wood near Oxford. Nine plots, each 2 yards square, were laid out over a small area of woodland carrying a heavy crop of bluebells. Each plot was divided into four sub-plots, each 1 square yard in area and each subjected to a different treatment. There were thus nine replications of each treatment, which were:

A. Control—untouched.
B. Picked just above ground level.
C. Pulled so that the white underground stalk was extracted.
D. Leaves and inflorescences picked off just above ground level.

It was realized later that it would have been more natural to have used heavy trampling in place of treatment D. The experiment was started in 1934, and repeated each year up to and including 1939.

Each year on two or more dates (Table 1), according to the state of the plots, which under varying conditions tended to come into full flower on different dates, the inflorescences from treatments B, C and D were removed and counted, and those on treatment A counted but left. It was realized too late to put it into practice that counting should have been augmented by weighing the inflorescences. The results are set out in Tables 2 and 3. The figures for the nine sub-plots are totalled for each treatment.

Table 1. *Dates of picking*

Year	Dates of picking	Year	Dates of picking
1934	10 May: Plots 1, 2, 7 14 May: Plots 5, 6, 9 18 May: Plots 3, 4, 8	1937	3 May: Plots 2, 3 11 May: Plots 1, 4, 5, 6, 7, 8, 9
1935	29 April: Plots 2, 9 3 May: Plots 1, 3, 4, 7 11 May: Plots 5, 6, 8	1938	28 April: Plots 1, 2, 3, 4 2 May: Plots 5, 6, 7, 8, 9
1936	9 May: Plots 1, 2, 7 15 May: Plots 3, 4 18 May: Plots 5, 6, 8, 9	1939	2 May: Plots 1, 2, 3, 7, 9 8 May: Plots 4, 5, 6, 8

From these results it is obvious that neither picking nor pulling had any deleterious effect on the production of flowers over a period of 5 years; there seems no reason to suppose that any effect would have appeared later. In view of the variations shown by B and C in relation to A in different years, their superiority over A on the 6-year total cannot be considered significant. In the absence of inflorescence weights, it can only be stated that there was no apparent difference in size between the inflorescence of treatments A, B and C. In the case of D, not only was there a big reduction in the number of flower spikes, but they were very much smaller. This reduction was scarcely apparent after 1 year's destruction, but was very evident the second year, and thereafter tended to get worse, though there was an unexplained partial recovery in 1939.

Table 2. *Numbers of inflorescences in each quarter-plot, and percentage changes in numbers from those counted in* 1934

Plot	Year	A		B		C		D	
			% change		% change		% change		% change
1	1934	173	—	205	—	170	—	186	—
	1935	173	0	239	+16·6	208	+22·4	154	−17·2
	1936	262	+51·4	242	+18·0	126	−25·9	191	+ 2·7
	1937	135	−22·0	140	−31·7	143	−15·9	43	−76·9
	1938	120	−30·6	136	−33·7	120	−29·4	21	−88·7
	1939	179	+ 3·5	189	− 7·8	251	+47·6	106	−43·0
2	1934	163	—	134	—	95	—	139	—
	1935	106	−35·0	155	+15·7	102	+ 7·4	83	−40·3
	1936	176	+ 8·0	108	−19·4	58	−39·0	110	−20·9
	1937	118	−27·6	137	+ 2·2	104	+ 9·5	32	−77·0
	1938	130	−20·2	112	−16·4	108	+13·7	40	−71·2
	1939	139	−14·7	111	−17·2	104	+ 9·5	84	−39·7
3	1934	135	—	128	—	112	—	128	—
	1935	155	+14·8	147	+14·8	145	+29·5	154	+20·3
	1936	142	+ 5·2	151	+18·0	150	+33·9	98	−23·4
	1937	112	−17·0	127	− 0·8	81	−27·7	74	−42·2
	1938	98	−27·4	140	+ 9·4	84	−25·0	30	−76·6
	1939	101	−25·2	108	−15·6	80	−28·6	38	−70·3
4	1934	163	—	168	—	161	—	171	—
	1935	193	+18·4	229	+36·3	234	+45·3	177	+ 3·5
	1936	220	+35·0	229	+36·3	236	+46·6	130	−24·0
	1937	140	−14·1	192	+14·3	139	−13·7	91	−46·8
	1938	131	−19·6	206	+22·6	152	− 5·6	65	−62·0
	1939	168	+ 3·1	163	− 3·0	177	+ 9·9	82	−52·0
5	1934	118	—	86	—	100	—	133	—
	1935	145	+22·9	138	+60·5	81	−19·0	80	−40·0
	1936	156	+32·2	70	−18·6	59	−41·0	64	−51·9
	1937	54	−54·2	22	−74·4	45	−55·0	28	−78·9
	1938	63	−46·6	56	−34·9	54	−46·0	20	−85·0
	1939	112	− 5·1	101	+17·4	121	+21·0	31	−76·7
6	1934	101	—	123	—	76	—	37	—
	1935	111	+ 9·9	100	−18·7	90	+18·4	146	+294·6
	1936	102	+ 1·0	144	+17·1	159	+109·2	115	+210·8
	1937	115	+13·9	136	+10·1	106	+39·5	96	+159·5
	1938	113	+11·9	62	+49·6	56	−26·3	57	+54·0
	1939	139	+37·6	133	+ 8·1	136	+79·0	55	+48·7
7	1934	144	—	142	—	157	—	149	—
	1935	85	−41·0	107	−24·6	144	− 8·3	126	−15·4
	1936	92	−36·1	95	−33·1	65	−58·6	77	−48·3
	1937	87	−39·6	106	−25·4	107	−31·8	68	−54·4
	1938	59	−59·0	73	−48·6	89	−43·3	53	−64·4
	1939	94	−34·7	88	−38·0	89	−43·3	40	−73·1
8	1934	126	—	124	—	125	—	94	—
	1935	86	−31·7	125	+ 0·8	108	−13·6	104	+10·6
	1936	169	+34·1	187	+50·8	164	+31·2	134	+42·6
	1937	134	+ 6·3	199	+60·5	124	− 0·8	90	− 4·3
	1938	123	− 2·4	105	−15·3	81	−35·2	59	−37·2
	1939	106	−15·9	204	+64·5	160	+28·0	85	− 9·6
9	1934	44	—	56	—	43	—	47	—
	1935	46	+ 4·5	39	−30·4	45	+ 4·7	30	−36·2
	1936	65	+47·7	70	+25·0	77	+79·1	49	+ 4·3
	1937	66	+50·0	82	+46·4	89	+107	39	−17·0
	1938	77	+75·0	91	+62·5	123	+186·1	39	−17·0
	1939	77	+75·0	88	+57·2	116	+169·8	69	+46·8

An analysis of variance shows significant ($p=0·05$) variation between years and between treatments. The only treatment difference which attains the 5 % level of significance is that between D and the average of A, B and C. The superiority of both B and C to A is suggestive but does not reach the 5 % level.

Thanks are due to Dr A. R. Clapham for his statistical treatment of the results.

Table 3. *Mean percentage changes from numbers counted in* 1934

	1935	1936	1937	1938	1939	Means
A	− 4·1	+19·8	−11·5	−13·2	+ 2·6	− 1·2
B	+ 7·9	+10·5	+ 0·1	− 0·5	+ 7·3	+ 5·0
C	+ 9·6	+15·1	+ 1·2	− 1·2	+32·5	+11·5
D	+20·0	+10·2	−26·4	−49·8	−29·9	−15·2
Means	+ 8·3	+13·9	− 9·2	−16·2	+ 3·1	—

The second author carried out a similar, but less extensive and exact, experiment at the Royal Botanic Gardens, Kew, from 1933 to 1940 inclusive. He wishes to acknowledge the help of several members of the Kew Staff, particularly Mr B. L. Burt. In 1933, four adjacent plots A–D, each 4 × 3 yards, were marked out in a fairly uniform stand of bluebells in the Queen's Cottage Grounds. In that year, and in each successive year until 1940, the plants in A were left untouched, those in B were picked just above ground level, those in C were pulled up so that the white underground stem was extracted, and those in D were trampled heavily, so that no leaf or flower stalk was left standing upright. Each year from 1934 to 1940 the plots were inspected at flowering time and an estimate made of the density and general condition of the plants; no exact counts or other measurements were taken. Apart from small and fluctuating variations, and a poor flowering season in all the plots in 1938 (probably due to the exceptionally cold and dry spring), the results can be summarized as follows: In plots A, B and C, the density and general condition of the plants remained more or less unchanged throughout the 8 years' treatment, whereas in plot D there was a progressive deterioration until, in 1940, there were only a few scattered plants, many of them not flowering. It will be seen, therefore, that this rather rough and ready test at Kew corroborates the results of the more exact experiments of the first author at Bagley Wood.

The absence of any ill effect from picking 'above ground', and the gradual diminution of vigour due to the removal or trampling of leaves are both results to be expected from a knowledge of the physiology and life history of bulbous plants. The prevention of fruit formation would naturally tend, if anything, to increase the vigour of the bulbs, while damage to the leaves would adversely affect the production of food by photosynthesis. Prevention of fruit formation would, of course, also prevent reproduction by seed, but in our experiments there were ample reservoirs of seed nearby to counteract any possible effect on numbers due to this cause, and the same would be true in nature, even with heavy picking. Woodhead (*The Naturalist*, February–March, 1904) has shown that, in nature, regeneration takes place by both seed and bulb increase but, as far as we are aware, there are no data available on the relative importance of the two methods.

The absence of ill-effects due to 'pulling' was perhaps not quite so obviously to be expected, as there is a very widespread belief that this form of picking does harm. It seems, however, from our experiments, that removal of the base of the flower stalk does no damage to the bulb.

It may be useful to sum up briefly what advice we suggest should be given to the public regarding the picking of bluebells. No harm can be done by moderate picking or pulling, preferably spread over a wide area rather than intensively in one spot, provided all unnecessary trampling on leaves is avoided. It is best to pick in a spot where the flowers are thinly scattered, as it is easier here to avoid trampling on the leaves.

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REVIEWS

Lectures on the Inorganic Nutrition of Plants. By D. R. Hoagland. 9 × 6 in. Pp. 226, with 28 plates. Waltham, Mass.: Chronica Botanica Co.; London: Wm. Dawson and Sons. 1944. $4.00.

A reprint of Prof. Hoagland's book has recently been prepared, which is in itself evidence of its usefulness to botanists during the four years since its first appearance. It is concerned with a branch of the subject where a formal text-book or perhaps even any attempt at a systematic monograph is not yet possible. Views are still in a very fluid state and new work is constantly appearing. The lectures referred to in the title were mostly included in a special series given by the author at Harvard and subsequently revised for publication. The author has, as he says, allowed himself a very liberal interpretation of the term nutrition, and has ranged from problems of the soil to those of intermediary metabolism. In all the chapters he has something useful and informative to say; but the real value of the book lies in those where he is condensing and summarizing the multifarious contributions of his own school and its collaborators to the study of plant nutrition. Far-reaching generalizations are not attempted; one feels that even apart from the special difficulties of the case they might be foreign to the nature of the author. There is, however, a steady advance from one established position to another, and a clear picture is gained of the important part played by this group of workers in the radical changes this subject has undergone since Prof. Hoagland first began his investigations. During this period many tools have become available for exploitation in their turn; and not the least interesting part of the story is that describing some of the earliest results obtained with radio-active tracers. It is with great regret that one learns that ill-health has latterly hindered the author's personal work; its inspiration is evident in that of numerous younger workers in the same field.

W. O. JAMES

Submicroscopic Morphology of Protoplasm and its Derivatives. By A. Frey-Wyssling. 10 × 6½ in. Pp. viii + 255, with 161 figures in the text. Amsterdam: Elsevier Publishing Co.; London: Cleaver-Hume. 1948. £1. 12*s*. 6*d*.

Submicroscopic morphology has a less formidable sound to biologists than physical chemistry. As a phrase it also serves to emphasize what the author calls the hierarchic system of structure at all levels of organization. It is therefore appropriately applied to the results of the application of physico-chemical methods to biological materials. This monograph has been prepared as a second edition of the author's already well-known text under the same title in German. As a result of 'war and post-war conditions' it is now published from Amsterdam in English.

The most notable event in its field since the publication of the first edition in 1938 has been the application of the electron microscope. The author indulges in a certain degree of nostalgia for the days before its advent and at times almost appears to regard it as something of a spoil-sport. The visible image does not always charm him so much as the same conclusion arrived at by ingenious indirect observation and accurate deduction. He is, however, consoled by the frequency with which the electron microscope has confirmed earlier findings; and also, one feels sure, by the severe limitations that yet restrict its use in biology.

The book starts with a modern account of colloidal systems, with concepts of phase and phase boundaries, and is particularly concerned with recent views on the structure of gels. The second part takes up the application to cytoplasm, nucleus, chloroplasts and erythrocytes in turn. A third section is devoted to protoplasmic products such as cellulose and starch grains. The English is almost always lucid and easy to read and very rarely descends to such horrors as 'de-mixing'. The numerous half-tones and diagrams are of great assistance to the text.

There has not previously been available to students in English so comprehensive a treatment of these subjects by an active and experienced worker. An important gap in English biological literature has been filled, and one may expect experience to show that it has been filled acceptably.

W. O. JAMES

Postglacial Forest Succession, Climate, and Chronology in the Pacific Northwest. By HENRY P. HANSEN. Pp. 130, with 114 text-figures and photographs. *Trans. Amer. Philos. Soc.*, New Series, vol. 37, Part 1. Philadelphia, 1947.

This paper presents the results of pollen analysis carried out on about seventy profiles in the states of Washington, Oregon, and part of Idaho. There is boldness in the way in which the author has attacked the problems of vegetational history over an area so large and so complex in its structure and climate. In spite of this complexity, the data exhibit a certain unity, and broadly speaking, they fall in with the general trends of climatic change which have been recognized in the post-glacial history of the more easterly regions of the U.S.A., and afford yet another confirmation of von Post's interpretation for the northern hemisphere in general.

Many of the profiles begin immediately above deposits associated with glaciation, and the author, after considering various estimates of late- and post-glacial time-scales, adopts the date of 1600 B.C. as representing roughly the time when these organic sediments began to accumulate. Layers of volcanic ash are found in the sediments and these are of much value in correlating levels in the various pollen diagrams. It seems, however, that the eruptions which caused these layers are not yet closely datable, and the proposed chronology is based on the acceptance of Antev's dating of the American phase of warmth maximum to the period 6000–2000 B.C. The most widespread layer of volcanic ash (probably derived from Glacier Peak in northern Washington) falls at or near the end of this phase, according to the author's interpretation of the pollen diagrams.

Superposed on the general agreement in the history of the area as a whole, there are strong and interesting differentiations shown by the various geographically distinct regions. In the Puget Sound region, the initial maximum of *Pinus contorta* gives place to dominance of *Pseudotsuga taxifolia*. The xerothermic phase is not well shown in the profiles of this region, but the subsequent return to cooler and moister conditions is held responsible for the rise of *Tsuga heterophylla* to its present important status. The early prominence of *Pinus contorta* is shown again in the Willamette Valley region of Oregon, and is indeed general throughout most of the other districts; here, however, *Pseudotsuga taxifolia* does not give way to *Tsuga heterophylla* in the upper layers, and the warmth maximum is evidenced by a clear maximum of grass pollen, followed by maxima of Chenopodiaceae and Compositae. Here too, as also in the Cascade Range of Oregon, *Pinus ponderosa* is and has been much more important than *Pseudotsuga taxifolia.* Only in the diagrams from the coastal strip is there a lack of any evidence of a definite climatic fluctuation. The marine influence has literally damped out any such effects, and complexities appear in the pollen diagrams which have to be interpreted in terms of the successional phases caused by the shifting sand-dunes so characteristic of this area.

There are many other points of interest in the paper such as the effects on vegetational succession which were produced by the thick pumice deposits from the eruptions of Mount Mazama, and the sudden maxima of *Larix occidentalis* which are taken to indicate the occurrence of severe fires. The data on the size-frequencies of the more important Pine pollens will be of value to the specialist, and a more general interest is served by the full account of present-day climate and vegetation types. Altogether there is no doubt as to the magnitude of the labour involved in this research and of the scientific insight which has gone to the choosing of sites for investigation. The author makes no attempt to hide the fact that his chronological correlations can be taken only in the widest sense; perhaps for this very reason his interpretations carry conviction, and it seems clear that his work forms a satisfactory basis for future detailed investigations in the region. V. M. CONWAY

Uredineana. Recueil d'Études Systématiques et Biologiques sur les Urédinées du Globe. Edited by A. L. GUYOT. Vol. II, 10 × 6½ in. Pp. 227, 3 text figures. Paris: Paul Lechevalier. 1946. 600 francs.

The first volume of Uredineana was published in 1938 and the reappearance of the second volume of this useful review is of considerable interest to students of the rust fungi. In this volume, the editor, Prof. Guyot, has assembled a number of papers describing systematic work on the Uredinales carried out during and immediately before the war, mainly on the continent of Europe.

The first four papers (two by M. Dupias and two by A. L. Guyot) are a series of notes on various species of the Uredinales found in different parts of France and in Algeria and Tunis, with remarks on their diagnosis and distribution. Some interesting observations are recorded in these papers which would repay further investigation. For example, *Puccinia polii* A. L. Guyot on *Teucrium montanum* in the mountain regions of south-east France is almost completely confined to altitudes between 1200 and 1500 m., although the host plant is much more widespread. A study of the various factors which might account for this limitation would probably yield interesting results. A number of new species are mentioned which are described more fully by Prof. Guyot, with Latin diagnoses, in the fifth paper of the volume.

In the sixth paper an account is given by A. L. Hagen of the rusts collected during the Norwegian Government Expedition to Greenland and Spitzbergen in 1933. No new species were discovered but a number of species were recorded which had not previously been found in Greenland.

The seventh paper is a clear and well-illustrated account by G. Kunnholz-Lordat of the morphological peculiarities of *Puccinia pilocarpi* Cooke on *Pilocarpus pinnatifolius* Lem. This rust is remarkable for the frequent separation of the two cells of the teleutospores and the tendency to atrophy of one or both of these cells. These observations are of considerable interest in connexion with Arthur's views on the relation between the genera *Puccinia* and *Uromyces*.

The eighth paper is a summary, by Prof. Eugen Mayor, of work carried out on the rust fungi in Switzerland from 1939 to 1945 and is of particular use and value to those of us who were cut off from continental literature during the war. The bulk of the work has been done by Prof. Gäumann and his colleagues at Zürich and consists mainly of thorough cultural and biometrical investigations of rusts occurring on a number of common plants, with a view to elucidating their taxonomy and life histories. Rusts occurring on *Carex* spp., *Solidago* spp., *Bupleurum* spp., *Epilobium* spp. and a number of other host plants have been studied in this way, and Prof. Mayor has made a careful experimental study of various heteroecious species of *Milesia* occurring in Switzerland.

Thorough cultural and morphological work of the kind carried out by these workers is the backbone of systematic Uredinology. In a group of plants of such simple morphology as the rusts, comparatively few characters are available for the differentiation of species and much reliance has to be placed on differences in pathogenicity and spore size and shape. It is particularly important that where pathogenicity is used as a distinguishing character, inoculation experiments should supplement field observations so that the host range of the species can be accurately determined and confusion caused by overlapping host ranges can be cleared up. An experimental comparison of the host ranges of *Puccinia bromi-maximi* Guyot described earlier in this volume as a new species by Prof. Guyot with those of other species infecting *Bromus* such as *Puccinia bromina* Eriks. and *P. bromicola* (E. B. Mains) A. L. Guyot would, for example, have been of considerable interest. As regards spore dimensions, it is surely essential, when these are used as criteria for the differentiation of a new species, that when this species is first described some idea should be given of the number of spores measured and of the statistical significance of the difference in size between them and the spores of related species. This appears to have been the general practice of Gäumann and his colleagues, but in the case of *P. bromi-maximi* and *P. vulpiae* described as new species by Guyot mainly on the strength of their spore dimensions, no such details are given, even in the first published accounts of these species in the fifth paper of this volume.

The volume ends with two bibliographical reviews and a bibliography of recent papers on the rust fungi. The first review is general and gives brief summaries of a number of papers published, mainly in Europe, during the war. As many of these papers were published in periodicals never readily available outside their country of origin these summaries are extremely useful to British workers. The second review is regional, giving references to records of species of the Uredinales occurring in a number of countries round the Mediterranean. There are also an appendix containing a number of additions and corrections to vol. 1 of Uredineana and good indices of fungus and host species.

The volume thus contains a great deal of information of considerable value to those interested in the systematics of the Uredinales. It is well produced and is carefully edited on the whole, but it is a source of irritation to the reader that all the contributors have not adopted the same system for the citation of their references.

M. R. GILSON

Der Lindenmischwald (*Tilieto-Asperuletum taurinae*) *des schweizerischen voralpinen Föhn- und Seenbezirkes, seine pflanzensoziologische und forstliche Bedeutung.* By Dr WALTER TREPP. Beiträge zur geobotanischen Landesaufnahme der Schweiz, Heft 27. Bern: Hans Huber. 1947. Fr. 11.50.

The *Tilieto-Asperuletum taurinae* is an association (using this word in the sense of the Zürich-Montpellier school) which reaches its best development on steep limestone slopes at levels of 430–650 m. around the Lake of Wallenstadt. *Tilia cordata* is dominant, *T. platyphyllos* and *Fraxinus excelsior* are constant species, while *Lonicera xylosteum, Corylus avellana, Cornus sanguinea, Rosa arvensis, Ligustrum vulgare, Viburnum opulus* and *V. lantana* are also species of high constancy. *Quercus* spp., *Acer* spp., *Ulmus glabra, Sorbus aria, Fagus sylvatica* and many other calcicole trees and shrubs are often present. Floristically the association is a rich one, a total of 48–65 species usually being present in a single sample of 10 × 10 m. The 'characteristic species' are *Tilia cordata, T. platyphyllos, Acer platanoides, Euonymus latifolius, Staphylea pinnata, Tamus communis, Asperula taurina, Viola mirabilis, Primula vulgaris* and *Cyclamen europaeum*, the last in great profusion and scenting the air in August. There are useful notes on the distribution and behaviour of these species in Europe.

Outside the region of Lake Wallenstadt the association reappears in similar situations, but without *Staphylea pinnata* around the Vierwaldstattersee, and, with the additional absence of *Cyclamen*, around the Lake of Brienz. All these regions enjoy a peculiarly favourable climate, largely attributable to the föhn wind which produces warm winters and springs compared with those of the northern Swiss lowlands, and reduces the period liable to frost. Moreover, the rainfall and humidity are relatively high, and the immediate neighbourhood of the three lakes has less fog (fewer than twenty days a year) than any other part of northern Switzerland.

Although typically a warmth-loving association of southerly slopes, there is a distinctive subassociation of cool aspects with abundant *Acer platanoides* and *Euonymus latifolius* and without *Tamus* or *Staphylea*. Other subassociations are described from less calcareous or slightly degraded soils.

Impoverished fragments of the association are scattered at various points to the north in the Swiss lowland, and stretch southwards down the Rhine valley to the neighbourhood of Chur and Bonaduz, while communities in which *Tilia* is either dominant or an important component occur in other parts of Switzerland, for example, the Lake of Geneva, the Lake of Thun, and some of the southern valleys of the Grison. A useful survey of these occurrences is given, with species-lists from several of them. Many are fragmentary relics only, and some of them are on 'Urgestein'; there are said to be 'almost pure lime woods, partly on Urgestein' in the neighbourhood of Innertkirchen. Many of these stands from the warmer, drier parts of the country have much more oak, especially *Quercus petraea*, than does the *Tilieto-Asperuletum*, and species which are regarded as characteristic of 'basiphil oakwoods', while the characteristic species of the association are practically absent.

The *Tilieto-Asperuletum taurinae* is regarded as a relict community which had its maximum distribution at the time of the post-glacial temperature-maximum, though no detailed evidence for this belief is given. At present it is largely confined to sites which are immature, and is dependent for its permanence on the continual rejuvenation of the soil on the steep slopes. On stable sites soil degradation eventually begins and acidiphil species enter—for example, *Quercus* spp., *Fagus, Betula verrucosa, Vaccinium myrtillus, Luzula sylvatica*, etc., and all stages can be found from this to beech forest, which is presumably the climax association of mature soils. On calcium-deficient rocks soil degradation may go much further, and the climax may be *Querceto-Betuletum*. It should, however, be noted that most of the lime-stands are relatively young and have been coppiced; Trepp makes little comment on this, and it is not clear to what extent the nature of the forest would change under continued high forest conditions. Natural regeneration of the lime rarely occurs in any quantity, and is usually on woodland margins, or in small clearings or gaps.

The paper contains some soil profile descriptions, mechanical analyses, pH measurements, and a useful discussion of the silvicultural potentialities of the association. E. W. JONES

The Diseases of the Flax Plant (Linum usitatissimum *Linn.*). By ARTHUR E. MUSKETT and JOHN COLHOUN, with a Foreword by REV. THE RIGHT HON. ROBERT MOORE, Minister of Agriculture for Northern Ireland. Cr. 4to, 10 × 7¼ in. Pp. 112, with 91 figures (38 in colour). Belfast: W. and G. Baird, Ltd. 1947. Price 21*s*.

The bulk of the flax seed required for the Northern Ireland crop was formerly imported, so the vast expansion in the acreage under fibre flax during the war demanded an unprecedented increase in the production of home-grown seed. The occurrence of epidemic outbreaks of stem-break and browning of flax in Northern Ireland in 1938 had led to the perfection of a rapid and effective technique for examining seed samples for the presence of the causal organism of the disease (*Polyspora lini* Lafferty), and other seed-borne parasites. This, the Ulster method, later proved readily adaptable for the evaluation of seed disinfectants. Thus it became possible, from 1941 onwards, to disinfect the whole of the wartime sowing flax seed produced in the United Kingdom—a measure which is estimated to have benefited the crop during the war period to the extent of some £2,000,000. This striking achievement represents only a part of the investigations, so largely original, of which the authors here present an account designed 'to make the subject intelligible to the lay reader as well as to the agricultural scientist and the farmer'.

The book is well documented, and is attractively produced and illustrated. The colour photographs should materially assist in the recognition of the several diseases. Although concerned in the main with fibre flax, the subject matter is largely applicable to oil flax as well: it provides a systematic treatment of the diseases of all types to which the flax plant is subject, including some which have not yet been recorded from the British Isles. There are four Appendices dealing respectively with the Ulster method of seed examination, seed disinfection, the production and certification of seed, and the growing of a healthy flax crop. The book as a whole will be welcomed as an authoritative treatment of its subject, and would appear admirably to have fulfilled the authors' purpose in writing it.

It remains to add that the publication has been sponsored by the Northern Ireland Flax Development Committee, which has arranged for the total proceeds to be used for furthering the work of the School of Agriculture in the Queen's University of Belfast. T. E. T. BOND

The Grasslands of Latin America. By G. M. ROSEVEARE. 10 × 7½ in., cloth. Pp. 291, with 9 plates and 15 figures (14 being maps). Aberystwyth: Imperial Bureau of Pastures and Field Crops (Bulletin 36). 1948. Price 20*s*.

Criticism is from time to time levelled at books and review articles which confine themselves to presenting a co-ordinated abstract of 'primary literature'—the data and conclusions of the original investigators—without propounding or supporting any particular view on controversial topics. As affecting students, lack of guidance in the use of such reviews may have its dangers. But there should be no quarrel on principle with works which in any way help to ease the otherwise gargantuan task of extracting knowledge from the vast, scattered mass of primary literature which now exists. To the average research worker, reviews of this kind are a godsend. In a foreword to this book they are strongly defended by Dr R. O. Whyte, who has directed the issue of many such reviews from the Aberystwyth bureau. If further vindication is required, this bulletin provides it, at any rate for a subject at the fact-amassing rather than the deductive stage.

Territorially, the treatment covers the American continent from Mexico southwards, plus Cuba. In subjects, which form the basis of the chapters, there is a big section (half of the total text) on the natural grasslands, classified as (1) good, (2) cool montane, (3) semi-arid, (4) hot savannahs. This is primarily an agronomic classification, which calls for little debate since these groups are immediately subdivided into examples which are treated independently. The succeeding chapters deal separately with a good range of topics: leys, plants good and bad (with a long list of browse plants), erosion and conservation, animal and plant health, management and improvement, research and government action. Throughout there is an admirable breadth of treatment, shown, for instance, in a concise pastoral history of Argentina. Indeed there are judicious excursions outside the strict concerns of grassland, such as an account of erosion control measures in Brazilian coffee plantations.

The author has sorted this information from a literature list of about 840 titles. In passing, one is struck—if no great linguist—by the ease of understanding titles in the Iberian languages; an encouragement towards the use of original papers when accessible. A compilation of this size and range needs a subject index, but there is none. This is the one shortcoming of serious proportions. The index of species, however, would have been still more useful if species altogether alien to Latin America could have been marked. Introduced species abound; yet names familiar in floras of other continents can by no means be taken to denote introductions. *Festuca ovina*, for instance, with other species familiar in Europe, is native in Patagonia; *Hyparrhenia rufa*, an African dominant, in Brazil and elsewhere. The photographs are helpful; one can be greedy for more than eighteen where so large and varied a territory is concerned. Most of the sketch-maps lack the customary convenience of a scale, but many readers will in any case require an atlas at hand.

Although the bulletin contains no theories of its own to call for critical comment, it is far from dry reading. In a continent ranging from the equator to 55° latitude (this the numerical equivalent of Northumberland, but with a climate more severe) and from lowland plains to the high Andes, every kind of major grassland habitat is represented. Even concentrating on the broader aspects of ecology, only a few examples can be given to illustrate the interesting material assembled.

To start in the south. Here some of the grasslands have quite a British character, not only in structure and in genera represented, but also in a good many species, native and introduced. Leguminous herbs, however, are often lacking; indeed they show a general deficiency in natural grasslands throughout the continent, although its leguminous flora is large. There seems to be a curious state of affairs in the southern Argentine-Chile borderlands. According to Hauman's description, the steep E.–W. climatic gradient is not accompanied by a commensurate change in vegetation. Nevertheless Davies, in the northern part of this region, noticed a compact change from subarid grass steppe to forest, apparently conforming closely to rainfall.

Another topic of general interest is raised in the account of the rich lands of Buenos Aires Province. Why are trees absent in this 'humid pampa', where they thrive when planted? In the experienced view of Prof. L. R. Parodi (who contributes a short chapter supplementing his many cited works), the compact and poorly aerated soil prevents access to the moist lower layers by roots of tree seedlings. Accepting this view together with Köppen's classification of the climate as one favouring forest, the pampa would be an edaphic climax *par excellence*.

Reference to Argentina prompts at least an aside on lucerne, for there is plentiful information in the chapter on leys about this important plant, whose acreage in Argentina is greater than in any other country in the world. It has contributed enormously to Argentine prosperity, and considerable research has been done on its biology and genetics. Nor is it neglected in other republics, although its success varies. In Chile and Peru its relative value to the country exceeds that in Argentina; in Uruguay, a mere 150 miles or so from its Argentine area (though not the chief part thereof), it behaves with regrettable obstinacy, apparently for edaphic reasons. The natural grassland of Uruguay has deteriorated under prolonged grazing; *Paspalum* species have been replaced by hard grasses of the genus *Stipa*. Yet despite these difficulties and a full share of others (mineral deficiencies, locusts), Uruguay remains a big exporter of meat and wool.

Northwards again into the tropics, the question of grassland status and tree-failure reappears in the savannas (to follow Burtt Davy's reasoned spelling of this word). Among these the Venezuelan llanos are especially vividly pictured by quotation from translated sixteenth-century Spanish to untranslated twentieth-century Journalese. Here there seems little question that the climate is discouraging to trees, but floods, fire and grazing are powerful factors too. The upland savannas of British and Dutch Guiana have been considered, though not agreed, to be a fire climax. Conflicting views exist about the effects of burning, on the llanos and elsewhere. The heaviest weight of opinion, including notably that of Dr H. Pittier of Venezuela, seems to be against the practice because of the selective elimination of nutritive species and the diminution of humus. There is no report of any attempt to settle the question by experiments, which might, of course, give different answers in different regions. This important problem of savanna country is closely matched in Africa. Another parallel—there must be many—is seen in the occurrence of different tree species on habitats differentiated by 'almost imperceptible elevations in the land'; in Africa this phenomenon seems fundamental in the determination of communities in savanna regions.

The importance of these great grazing lands should need no emphasis in our food-conscious era. There is ample evidence of keenness in a number of the republics to use them to the best advantage through scientific study of their problems. In these efforts the bulletin will be of obvious usefulness. Also, however, it reveals much with a bearing in the British Commonwealth; the parallels just mentioned with African conditions are merely examples in a particular sphere. The Imperial (henceforward Commonwealth) Bureau has, therefore, made a valuable addition to its output in publishing this product of Miss Roseveare's able and arduous work, for which there will be a debt to her in many lands.

J. F. HOPE-SIMPSON

Biology of Pathogenic Fungi. Edited by WALTER J. NICKERSON, with a Foreword by J. G. HOPKINS. Pp. xx+236, 9 plates and 31 text-figures. Waltham, Mass., U.S.A.: the Chronica Botanica Co.; London: Wm. Dawson and Sons, Ltd. 1947. Price $5.

Although fungi are more important as pathogens of plants than of animals, the discovery of the part they play in the muscardine disease of silkworms and in ringworm of man antedated by a few years their recognition as agents of plant disease. For twenty years following Gruby's classical work on human ringworm in the early eighteen-forties there was much speculation as to the possible implication of fungi in other diseases which afflict man. With the rise of bacteriology medical mycology suffered eclipse but during the closing years of the century interest revived, largely due to the researches of Sabouraud whose name still dominates the subject. Unfortunately, medical mycology has had few firm contacts with general mycology. Much unnecessary confusion has been introduced by medical men into studies on pathogenic fungi and the rare excursions of non-medical mycologists into the medical field have not been characterized by the happiest results. During the last two decades there has been a welcome change. Trained mycologists, particularly in the United States, have studied medical fungi in the light of modern knowledge and collaborated with medical men in clinical investigations. The book under review is symptomatic of this new approach. More than half the authors of the series of well-documented reviews which make up the volume are mycologists by training. The topics dealt with are diverse and the book is given a unity more by its covers than its contents. It is, nevertheless, a most useful addition to the literature of medical mycology and may be recommended for the library list of any botanical department.

Several important diseases are treated authoritatively from a mycological angle—coccidioidomycosis by Emmons, chromoblastomycosis by Carrion and Silva, torulosis by Lodder and De Minjer—and D. S. Martin surveys the geographical distribution of systemic fungus infections, but it is the chapters on nutrition and metabolism that general botanists will probably find most stimulating. Among these is one by Miss Benham summarizing researches by herself and others on the peculiar growth requirements of *Pityrosporum ovale.* This organism, which is found associated with dandruff and is at most only weakly pathogenic, is much more exacting than are more highly pathogenic species. There are also sections on the production of pigments, polysaccharides, and lipoids by pathogenic fungi by J. W. Williams, R. C. Peck, and by Nickerson who also discusses his pioneer studies on the respiration of these forms.

In conclusion it may be noted that while the editorial latitude on questions of nomenclature accurately reflects the diversity in current usage, the unwary reader may not realize that all the information in the book on the cause of torulosis, for example, can only be obtained from the index by consulting three unrelated binomials.

G. C. AINSWORTH

NOTICE

A proof of Prof. Geitler's revision of the Cyanophyceae in the *Natürliche Pflanzenfamilien* (vol. 1, 1942), most of the copies of which were destroyed during the war, has been sent by the author to Prof. Fritsch (at the Botany School, Cambridge). He will be prepared to loan it for short periods to anyone who may be interested.

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OBSERVATIONS ON THE ENZYMATIC ACTION OF MAPLE AND BIRCH SAPS

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(With 3 Figures in the text)

INTRODUCTION

In 1938 the Canadian authors Bois & Nadeau (1938*a*, *b*) published the results of their researches on the enzymes present in the sap of the sugar maple, *Acer saccharum* Marsh. The conclusion to which they came may be summarized as follows: The sap contains two amylolytic enzymes with different pH and temperature optima. The first, which they call sucrogène-amylase, is said to form cane sugar from starch. According to them it has an optimum pH of about 6·6 and an optimum temperature of about +8° C. The second enzyme, the cellobiogène-amylase, is said to form cellobiose from starch and to be characterized by a much higher optimum temperature (40–50° C.) and by an optimum pH of 4·8–5·3. In more recent publications Bois and collaborators (1942; 1945) have considered the enzymes of the potato and those present in birch sap. In the former the presence of sucrogène-amylase is again claimed; in the latter the presence of cellobiogène-amylase and of a glucose-forming enzyme.

Reading these publications one is led to believe that the formation of cane sugar from starch is a *direct* one. Should this point of view prove to be correct, a complete reversal of theories of the structure of starch would result; for the usual conception is that starch is built of chains of linked maltose molecules. These maltose molecules, in their turn, consist of two gluco-pyranose units. Should sucrose as such be present in starch, then fructo-furanose rings should be found as well as gluco-pyranose rings. The attractiveness of the reported findings is that they might lead to a better understanding of the very easy conversion, in the living plant, of starch into sucrose and vice versa. The alleged production of cellobiose would provide us with the 'missing link' between cellulose and starch chemistry and would be intelligible from a teleological standpoint; for the cellobiogène-amylase is said to be prominent in the tree saps in late spring when the buds are about to open and need cellobiose as a building material for their growing cell walls. The tacit assumption here is that free cellobiose can indeed be used by plant cells for this purpose. Hestrin, Aschner & Mager (1947), working on cellulose synthesis by *Acetobacter xylinum* preparations, found no evidence for this. On the other hand, Haller (1945), comparing the results of treating starch granules with iodine-water and with potassium iodate solutions, concluded that there was at least a framework of a cellobiose polymer in the outer layer of the granules. In view of this conflicting evidence, and of the fact that Hassid, Doudoroff & Barker (1944) have meanwhile effected a synthesis of sucrose, it seemed worth while to reconsider the whole problem critically, and this is the aim of the present paper. The experiments described were begun as early as 1939 and were continued for four years. In order to confirm them and to check the results reported by Bois and collaborators, new experiments were carried out, and many of the old ones repeated, in 1947.

METHODS

(1) *Extraction of the tree saps.* Four species of maple (*Acer campestre, A. dasycarpum, A. negundo* and *A. saccharum*), five of birch (*Betula lenta, B. populifolia, B. papyrifera B. pubescens,* and a hybrid between the last two), hornbeam (*Carpinus betulus*) and a hop-hornbeam (*Ostrya* sp.) were used in the investigation, but most of the experiments were carried out on *Acer dasycarpum* Ehrh. After the first few preliminary experiments the so-called American method described by Bryan, Hubbard & Sherwood (1937), involving the use of a metal spout, was adopted for collecting the sap. In order to avoid a possible influence of metallic ions on the amylolytic enzymes, the metal spouts were later replaced by wicks of clean cotton which filled the holes in the trees. The sap dripping down these wicks was collected in glass vessels containing some disinfectant (toluene and/or chloroform). In the experiments on birch, branches were sometimes cut off and Erlenmeyer flasks attached to the stubs in such a way that the necks were covered by canvas cuffs to avoid contamination or dilution by rain water. The utmost care was taken to prevent yeast infection or contact with human hands or saliva. Every morning and evening the sap was collected and the cut surfaces and wicks were examined and, if necessary, renewed. Concentration of the sap was effected by evaporation *in vacuo* or freezing. The latter method was used exclusively in 1947. The concentrated saps keep very well under a layer of toluene in the refrigerator, and the unconcentrated birch sap does not lose its amylolytic power after two months at a temperature not exceeding 15° C. To get information on the effect of weather on sap production, temperature records were kept. The pH and enzymic activity of the saps were followed throughout the whole season.

(2) *Biological sugar determinations.* For most experiments use was made of the microfermentation method devised as early as 1913 by van Lutsenburg Maas & van Iterson (1915).

The method is based on the same principles as the well-known one of Kluyver (1914): the amounts of the various fermentable sugars present in a mixture are computed from the quantities of carbon dioxide produced by yeasts which selectively ferment monoses, monoses and sucrose, and monoses, sucrose and maltose respectively. Very small quantities of sugar (0·1–3·5 mg.) in drops of fluid (0·010–0·100 ml.) may be determined. As a rule 0·050 ml. of a 3 % solution (1·5 mg. of dry matter) gives the best results. A full discussion of the advantages and disadvantages of fermentation methods in general and of the micro-saccharimeter in particular is given in my Thesis (Meeuse, 1943).

(3) *Chemical methods for sugar determination.* A rather extensive study of the various methods for estimating sugars by their reducing power was made (Meeuse, 1943). The principal method used was that described by Harding & Downs (1933) and also advocated by van der Plank (1936).

Sucrose was estimated by measuring the reducing power of the solution before and after inversion by means of invertase or hydrochloric acid. For a comparative discussion of the merits of the two modes of inversion see Lehmann (1931) and van der Plank (1936).

Fructose. (*a*) The method of Sieben consists in the destruction of fructose by heating the sugar solution for a few hours with weak hydrochloric acid in a boiling water-bath. Glucose remains practically unchanged. Particulars are given by Lehmann (1931).

(*b*) In the method of Kruisheer-Kolthoff the aldoses in solution are oxidized by means of hypoiodite: the remaining reducing power is due to ketoses. The aldoses are found by taking the differences between the values before and after hypoiodite treatment. Van der Plank (1936) gives a micro-method based on this principle.

(*c*) Roe's colorimetric method (1934) is a modified Seliwanoff method. The fructose develops a red colour when heated with resorcinol and hydrochloric acid.

Maltose. The best results were obtained by utilizing the Barfoed principle. The reduction value is estimated first with the 'acetate reagent', which does not oxidize bioses, and then with the 'tartrate reagent' (e.g. Fehling's solutions I and II) which attacks maltose and other reducing bioses as well. The maltose is found by difference. Zerban & Sattler (1938) have improved upon the original method.

Starch. Hanes's method was used, as modified by Hassid, McCready & Rosenfels (1940). The material, e.g. dry root-powder, is extracted with boiling acid alcohol so that the sugars are removed while the starch is merely solubilized. Then the soluble starch is extracted with hot water, and, for the most part, transformed into maltose by means of amylase. The maltose is determined by its reducing power.

(4) *Osazone formation.* We found the procedure followed by Bois & Nadeau (1938*a*) to separate cellobiosazone from glucosazone (hot filtration to separate the glucosazone and cooling down the filtrate to cause sedimentation of cellobiosazone) to be ineffective. We therefore tried to obtain the cellobiosazone by first eliminating sucrose and other fermentable sugars by means of yeasts.

The method was to allow a thick suspension of the almost pure Delft 'Koningsgist' to act on the solution for 6–12 hr. at 35° C., as in the micro-saccharimeter technique of van Lutsenburg Maas and van Iterson (1915). Sometimes sodium bicarbonate was added to keep the pH constant and to ensure complete fermentation. After this incubation the yeast was spun down in a Laval separator. The clear fluid obtained was boiled and filtered to eliminate small amounts of protein, and concentrated by evaporation at low pressure. The procedure indicated here was varied sometimes, by extending the fermentation time to 18 hr. or by precipitating the dextrins with alcohol. In a few cases the fluid finally obtained was purified with the aid of ion-exchange adsorbents in order to remove salts. The osazone formation itself was usually carried out under the conditions indicated by Smits van Waesberghe (1941). For maltosazone this author recommends a very gradual cooling by simply turning out the flame under the water-bath and allowing the mixture to stand in this during the night. The quantities used were usually 4 g. of phenylhydrazine hydrochloride and 6 g. of sodium acetate per 50 ml. Control experiments on weak solutions of cellobiose showed that the sugar was not appreciably attacked by the treatment, so that it could be recovered with a yield almost equal to that obtained from the fresh pure solution.

(5) *Identification of sugars by their inhibition of enzymatic reactions.* For the identification of the sugars forming part of certain glycosides Ter Meulen introduced the following method. To various samples of the solution of a glycoside which was being enzymatically split into sugar and aglycone, he added various sugars. The sugar exerting the strongest inhibitory action he considered to be the one produced by the hydrolysis and therefore present in the glycoside. It will be clear that the method is based on the reversibility of enzyme reactions.

The difficulty in applying Ter Meulen's principle to the enzymes present in maple sap is that this sap already contains sucrose. Dilution or dialysis of the maple sap with the aim of reducing its sugar content to negligible amounts leads to too heavy a loss in enzyme activity, initially very low in all our experiments. A more satisfactory technique was to use gelatine-starch plates into which the various sugars were introduced in fairly high concentrations, e.g. 10 %. If a drop of maple or birch sap is put upon such a plate, the small sugar molecules present in it will rapidly spread all through the plate, as may easily be demonstrated on plates of pure gelatine by means of sugar reactions. If, then, the drop is made very small and the plate large, the concentration of sugar present in the sap will fall to a very low value. The iodine reaction is examined after a few days. It is useful to run control experiments using well-known enzyme preparations such as saliva, malt amylase or taka-diastase.

Beside the plate experiments others were carried out in the ordinary way, in test-tubes. Instead of the original saps alcohol precipitates obtained from them were sometimes used. The experiments were carried out at various temperatures and various acidities in order to test the possibility of more than one enzyme being present.

(6) *Wijsman's test to show the presence of α- and β-amylase in a mixture.* The experiment by means of which Wijsman (1889*a*, *b*) demonstrated in a both simple and conclusive way the existence of the two enzymes, α- and β-amylase, in malt extract is still too little known. Since an excellent description of the method has been given by Hanes (1937), we shall here only indicate the principle involved. By diffusion into a starch-gelatine plate subsequently treated with iodine, malt extract is shown to contain two enzymes with different diffusion velocities. The one diffusing more rapidly leaves behind a substance which still assumes a violet or reddish colour with iodine.

This enzyme is sometimes called an erythro-amylase, following Beijerinck, who used to speak of erythro-diastase. Unfortunately, when this erythro-amylase is allowed to attack a paste of untreated potato starch instead of the soluble starch, the colour change is hardly perceptible to the naked eye (A. Ph. Weber, oral communication). It is, therefore, preferable to use the name β-amylase, since it forms β-maltose. The slowly diffusing enzyme, an α-amylase, breaks starch down to products no longer giving a colour with iodine, whence the old name leuco-amylase. Heating the malt extract to 70° C. causes the ring to grow narrower, and according to Wijsman heating for 10 min. eliminates it almost completely. A weak acidification of the malt extract by means of lactic acid causes the ring to become relatively broader. The two enzymes are then differently susceptible to high temperature and acidity.

(7) *The use of luminescent bacteria for sugar identification* was also introduced by Wijsman, who took advantage of the fact that some of these bacteria, having exhausted the medium of the substances essential for their luminescence, resume emission when certain sugars are added. According to Wijsman the action of β-amylase on starch-gelatine-fishbroth plates causes luminescence through the rapid formation of free maltose, whereas α-amylase is said to have no or very little effect since it forms initially only dextrins. Weber (1941) was able to show that the starch concentration is very important. If sufficiently high a positive reaction is also given by α-amylase, so that the method is not as specific as Wijsman believed.

In our experiments Wijsman's bacteria were grown on starch-gelatine-fishbroth plates at a moderate temperature. When the luminescence had ceased the sugars glucose, fructose, sucrose, maltose, galactose, lactose and cellobiose were tested for their power to cause resumption. Cellobiose and sucrose were negative even when offered in the solid state, but maltose, fructose and glucose gave a positive reaction. We had, therefore, always to free our enzyme preparations from these sugars beforehand, and worked with concentrated maple juice (almost pure cane sugar) and washed alcohol precipitates from birch juice.

(8) *Simple test to demonstrate phosphorylase activity.* Under the influence of the enzyme phosphorylase an equilibrium is obtained between glucose-1-phosphate on the one hand, and free phosphate and starch on the other. This may be utilized in order to demonstrate the presence of the enzyme.

The glucose-1-phosphate is prepared by Hanes's method (1940), in the form of the di-potassium salt. To 50 ml. of bacteriological agar, 0·5 g. of the substance is added, the mixture is boiled until the froth disappears completely on cooling, and is then poured into Petri dishes. After cooling, drops of the fluid to be tested are allowed to act upon the plates at a temperature of about 35° C. After 1–6 hr. the plates are 'developed' by means of a weak solution of iodine in potassium iodide. If a strong phosphorylase is present, e.g. in potato press-juice, we shall observe a ring of blue-staining synthetic starch round a colourless centre, the latter due to the action of the ordinary amylases which usually accompany the phosphorylase. The advantage of the method is that one glance suffices to give an idea of the relative quantities of the two kinds of enzymes. If the amount of amylase is great the ring will not be blue but violet or even reddish; if very high the ring will be completely absent, and the method therefore fails.

(9) *Demonstration of cellobiose.* By means of cellobiase (β-glucosidase) cellobiose can be converted into glucose, which can then be estimated by fermentation or reduction methods. The classical material from which to prepare cellobiase solutions is the kernel of the apricot. By grinding fresh kernels in a mortar with water and filtering, a clear yellowish fluid is obtained which brings about glucose formation when incubated at 50–55° C. with a cellobiose solution, a small quantity of phosphate buffer and a few drops of toluene. Since fresh kernels are sometimes difficult to obtain, other sources are preferable. After many experiments with snail enzymes and takadiastase we came to the conclusion that emulsin is the most satisfactory source of cellobiase. In some emulsin preparations the amylase and maltase activities are very low and the β-glucosidase activity very strong.

(*a*) *Preparation of emulsin and amygdalin from bitter almonds.* The material is ground and pressed. For the preparation of amygdalin the greater part of the press-cake is extracted with light petrol and subsequently with alcohol. Addition of ether to the alcohol extract precipitates the amygdalin. To obtain the emulsin another part of the press-cake is extracted with ether at a temperature not exceeding 40° C. After this it is extracted a few times with alcohol at 40° C., and then the residue is extracted with cold water. Addition of alcohol to the water extract precipitates the emulsin. To test its β-glucosidase activity the emulsin is incubated with the amygdalin and water in the presence of a disinfectant. The splitting of the glycoside is apparent from the strong reducing power of the solution after incubation and from the strong smell of hydrocyanic acid.

(*b*) *Preparation of cellobiose.* The octacetate was prepared first, the most convenient method being found to be that of Friese & Hess (1926, 1927): the alternative is that of Spencer (1929). To prepare free cellobiose from the octacetate 5 g. of the latter are moistened with alcohol, and 25 ml. of alcoholic caustic potash (15 %) are added to the mixture. Filter under suction, wash with alcohol, dissolve in water, and neutralize with acetic acid. Concentrate the filtrate by evaporation, add alcohol ether, and dissolve the resulting syrup in water. Evaporate and add alcohol for recrystallization.

(*c*) *The testing of yeasts for their cellobiose-fermenting power.* A cellobiose-fermenting yeast would be a powerful tool in our research and therefore some thirty species and varieties were examined for this property. Since it was found that in bacteria there is a correlation between cellobiose-splitting power and power to decompose glycosides such as arbutin and aesculin, special attention was paid to yeasts possessing the latter capacity: *Candida tropicalis* (A. Cast.) Berkhout and its vars. *lambica* (Harrison) Diddens & Lodder and *rhagii* Diddens & Lodder, *C. pulcherrima* (Lindner) Windisch, *C. mesenterica* (Geiger) Diddens & Lodder, *C. pseudotropicalis* (A. Cast.) Berkhout, *C. humicola* (Daszewska) Diddens & Lodder, *C. reukaufi* (Grüss) Diddens & Lodder, *C. macedonicensis* (A. Cast. & Chalmers) Berkhout, *C. pelliculosa* Red., *C. intermedia* (Cif. & Ashf.) Langeron & Guerra, *C. tenuis* Diddens & Lodder, *Hansenula suaveolens* (Klöcker) Dekker (*Pichia suaveolens* Klöcker), *H. anomala* (Hansen) Sydow and its vars. *sphaerica* (Nägeli) Dekker, *longa* Dekker, *robusta* Dekker and *productiva* (Beijerinck) Dekker, *H. saturnus* (Klöcker) Sydow, *H. javanica* (Groenewege) Dekker, *H. schneggii* (Weber) Dekker, *Pichia polymorpha* Klöcker, *Zygopichia farinosa* (Lindner) Klöcker (*Pichia farinosa* (Lindner) Hansen). In the micro-saccharimeter of van Lutsenburg Maas & van Iterson (1915), however, no fermentation of cellobiose by these yeasts could be demonstrated. Nor could it be demonstrated with *Candida robusta* Diddens & Lodder, *Saccharomyces cerevisiae* Hansen, *S. fragilis* Joergensen, *Schizosaccharomyces pombe* Lindner, *Zygosaccharomyces lactis* Dombrowski, *Torula monosa* Kluyver, *T. lactosa* Kluyver, *T. cremoris* Hammer & Cordes, *Torulopsis dattila* (Kluyver) Lodder, *T. kefyr* (Beijerinck) Lodder, *T. sphaerica* (Hammer & Cordes) Lodder, some yeasts kept in the Laboratory for Technical Botany under the names 'Unterhefe U' and 'lactose yeast', and finally two strains of *Endomycopsis fibuliger* (Lindner) Dekker and its var. *lindneri* (Saito) Dekker. Though no tests were made to investigate the possibility of cellobiose fermentation under aerobic conditions, the negative results obtained suggest that cellobiose is not found in the free state in tree saps, since if it were one would expect some of the wild yeasts to ferment it.

(10) *Adsorption experiments.* The preparation of the aluminium-hydroxide Cγ used here was carried out according to the instructions of Grassmann (1928).

Experimental results

Influence of weather on quantity of sap. Fig. 1 shows that night-frost has a stimulating effect on the flow of *Acer dasycarpum*. On birch no such influence could be observed.

pH *of the saps.* In 1947, the pH of the saps was measured every day to check the statement by Bois & Nadeau that the pH falls as the season advances (in *Acer*). Indeed the first sap, obtained on 16 January, had the highest pH, viz. 7·1. On 5 March, after a severe frost period, it was 6·5, on 14 March 6·2–6·3. It remained constant from this date onwards till the end of the bleeding-season (29 March). It will be appreciated that this pH is closer to the alleged optimum for 'sucrogène-amylase' than to that for 'cellobiogène-amylase'. In birch, where the 1947 season lasted from 23 March till 10 April, the pH remained 6·1–6·2 all the time, with the exception of 2 April, when it was 5·8.

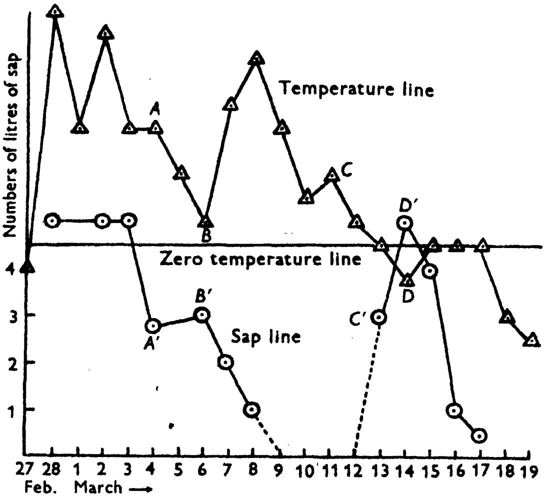

Fig. 1. Relation between 8 a.m. temperature and sap flow of a specimen of *Acer dasycarpum* for the end of the sap season of 1941. After the cold period of February 27 there follows stable, rather mild weather; there is a continuous and slowly decreasing flow. After the drop *AB* there follows a rise in flow *A'B'*. During the subsequent spell of stable and mild weather, the sap flow is reduced to nought. The drop in temperature *CD* corresponds to the rise in sap flow *C'D'*.

Optimum temperature for starch hydrolysis. Taking the disappearance of the iodine reaction as a measure for the hydrolysis, we found the optimum temperature to be about 50° C. for both birch and maple saps. Various concentrations of starch were used so that the times necessary for complete disappearance of the iodine colour also varied considerably, yet there was hardly any shift in the optimum temperature. So there is no great loss of enzymic activity at 50° C. This favours the view that a (thermoresistant) α-amylase is at work.

Optimum pH *for starch hydrolysis.* Again the disappearance of the iodine reaction was used to measure the starch breakdown. Sörensen's acetate and phosphate buffers were used. The experiments were carried out at temperatures varying from 1 to 50° C. For the lower temperatures we sometimes used saps concentrated by freezing in order to speed up the breakdown process. In spite of this, very low concentrations of starch had to be used here. The optimum pH was in the vicinity of 5·2–5·6 for both maple and birch, for all temperatures examined and for both the beginning and the end of the season. So no indications for the presence of more than one enzyme can be collected from these experiments alone.

The results of the experiments in which the hydrolysis was followed by measuring the reducing power of the starch-sap mixtures are in good agreement with those mentioned already. As an example we shall give only the hydrolysis of starch by birch sap at 35° C., under the following experimental conditions: 10 ml. of a 4% starch solution (soluble starch, Kahlbaum) are added to 10 ml. of birch sap, 2 ml. of M/2 acetate buffer and a few drops of toluene. After 2 days of incubation 1 ml. of the mixture is diluted to 101 ml. with water. 5 ml. of the fluid obtained are used to measure the reducing power by the method of Harding & Downs (1933). The values found are:

pH	Reduction in mg. of maltose
3·6	0·045
4·0	0·352
4·4	0·520
4·8	0·597
5·2	0·658
5·6	0·689

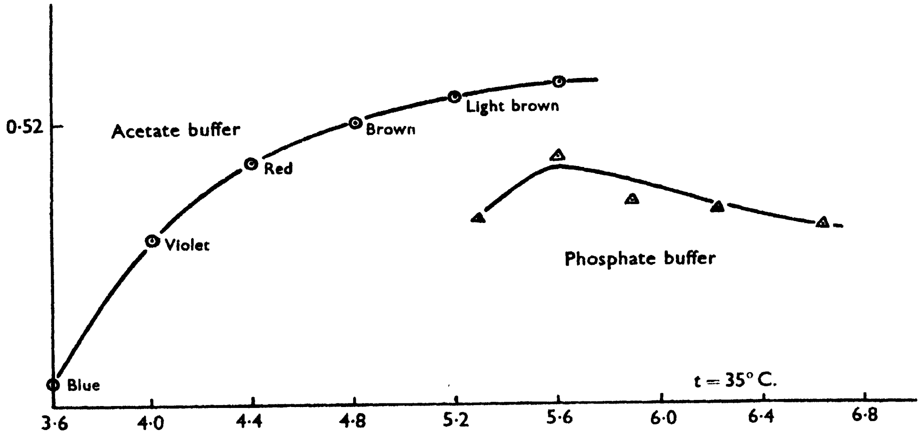

Fig. 2. Optimum pH for the hydrolysis of soluble starch by birch sap, at 35° C. Abscissa: pH values. Ordinate: reduction in mg. of maltose found with the aid of the Harding & Downs method (1933).

The results of this experiment, and of a similar one where phosphate buffer was used, are plotted in Fig. 2. It is evident that there is a broad zone of optimal activity. This, together with the fact that the same optimal pH is found when colour with iodine and reducing power are used as measures of activity, gives us another argument for the presence of α-amylase. Yet some other enzyme must be present as well, for in the experiment where the pH was 5·2, the iodine colour is still brown, whereas the reducing power is about 35% of the theoretical maltose; in α-malt-amylase the reducing power is only 33% when the achroic point has been reached already.

Result of Wijsman's test. In view of the extreme weakness of the enzymes present in maple and birch saps they were concentrated by freezing methods. Drops of the fluids obtained were put on gelatin plates containing starch in 0·1, 0·25 and 0·5% concentrations, and the enzymes were allowed to work at temperatures only slightly above zero point. Some of the plates were treated with iodine after 5, some after 10 and some after 15 days. These experiments revealed the presence of (at least) two enzymes just as in Wijsman's results. In birch sap the coloured ring was narrower than in maple sap.

Maltase activity of birch sap. 5 ml. of a 15% solution of maltose-hydrate was mixed with 10 ml. of birch sap, 5 ml. of phosphate buffer and a few drops of toluene, and incubated at 34° C. After 7 and 14 days, 1 ml. was taken out of every flask, diluted to 101 ml. and the reduction power of 5 ml. of the fluid obtained was measured with the method of Harding and Downs. The results were (see Fig. 3):

pH	Reduction as mg. of glucose, after	
	7 days	14 days
5·29	0·23	0·44
5·59	0·20	0·37
5·91	0·19	0·34
6·24	0·14	0·27
6·64	0·08	0·19

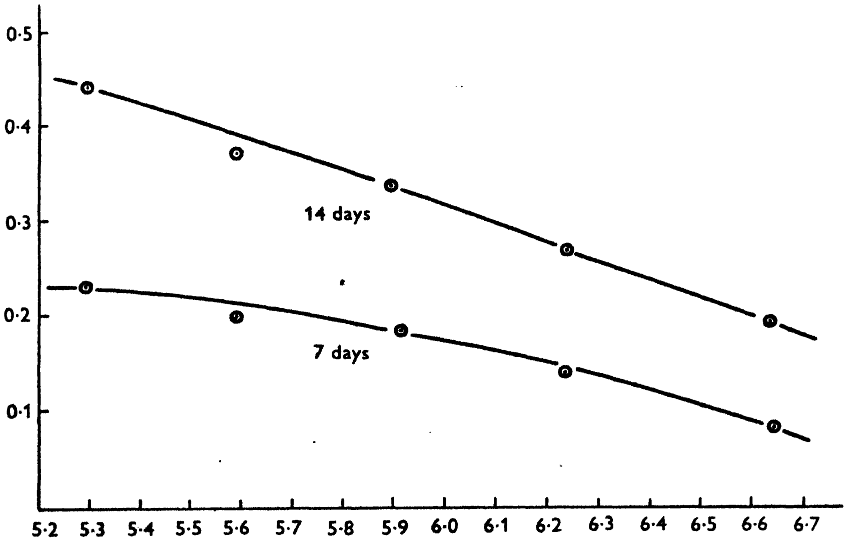

Fig. 3. Optimum pH for the maltase activity of birch sap, at 34° C. Abscissa: pH values. Ordinate: reduction in mg. of glucose found with the aid of the Harding & Downs method (1933).

These results were confirmed by carrying out fermentation experiments. In 1947, for example, 50 ml. of birch sap were incubated with 2 g. of maltose-hydrate and a few drops of toluene and incubated at 34° C., without buffer, for 19 days. After elimination of the toluene, *Torula monosa* showed the presence of 2·92% of monose in the fluid, whereas the monose concentration in the birch sap was only 1·52%. Control tests with *Saccharomyces cerevisiae* and *Torulopsis dattila* showed satisfactory agreement with the amounts of theoretical maltose and of monose respectively.

The optimum temperature for maltase action was found to be 34° C. At 50° C. there is probably destruction of the enzyme since the experiments take so long a time.

Slight maltase activity was also found for the saps of *Carpinus* and *Ostrya*. For maple sap the presence of maltase could not be ascertained.

The invertase and cellobiase activity of the saps was found to be negligible (absent) in all cases.

Formation of maltose through action of maple and birch saps on starch

(*a*) *Birch sap*. 50 ml. of sap was mixed with 5 ml. of phosphate buffer (pH 5·2) and 50 ml. of a 5% soluble starch solution. After 2 days of incubation at 50° C. the iodine reaction had disappeared. After 3 days the mixture was boiled and the sugars were estimated by fermentation. The initial concentration of monoses in the mixture was 0·52%, and the final concentration 0·97%. Maltose, only present at the end, reached 0·385%. The yield of monose and maltose together was about 38% of the starch.

(*b*) *Maple sap*. 50 ml. of concentrated sap were mixed with 50 ml. of phosphate buffer (pH 5·6) and 50 ml. of a starch solution, so that the initial concentration of starch was 8·57%. After 3 weeks' incubation with toluene at 50° C. there was a white precipitate resembling cotton-wool, which, however, did not give an iodine reaction. After boiling and cooling, a bright blue colour was visible on adding iodine. The fluid was analysed with the aid of the micro-saccharimeter, and it contained 1·46% of maltose, equivalent to 17% of the starch originally present. When the boiled fluid was cooled and mixed with fresh sap, a white precipitate was again formed after a few days, though the quantity was now very small. On boiling and adding a minute quantity of iodine solution there was again a bluish grey tinge. The observed phenomenon may be one of retrogradation; it is a well-known fact that weak amylolysis favours this process. The reappearance of the iodine colour on boiling must be some spontaneous resynthesis. It has also been reported by Naegeli for 'skeletons' of starch granules, and by Smits van Waesberghe for mixtures like ours. No attempts were made to investigate the phenomenon further, but in order to avoid the formation of a precipitate as described, a number of experiments were carried out on amylopectin, prepared according to Haworth, Peat & Sagrott (1946). The retrogradation was negligible in this case, and the formation of maltose in small quantity could again be demonstrated. It was found that the resistance of the amylopectin to amylolytic attack by maple and birch sap was greater than that of the soluble starch used in the earlier experiments.

Formation of dextrin through action of the saps on starch

400 ml. of maple sap were mixed with 50 ml. of buffer (pH 5·6), 50 ml. of water and 20 g. of soluble starch containing 10% of water. After 10 days' incubation at 50° C. fermentation tests showed the mixture to contain 18% of the theoretical amount of maltose. 25 ml. of the fluid were mixed with 1 ml. of a saliva solution which was now allowed to work at 40° C. Samples were taken after 3·5 and 5 hr. and analysed with the aid of the micro-saccharimeter. After 3·5 hr. 77·8%, and after 5 hr., 82% of the theoretical amount of maltose was found. The conclusion must be that the action of the maple sap did not lead to the formation of appreciable amounts of cellobiose; for it is a well-known fact that saliva alone, working on starch not previously treated with enzymes, is not able to give much more maltose than found by us here.

Similar results were found for the action of birch sap. We shall here give only the result of one experiment with this fluid. Equal volumes of a 3% starch solution and fresh birch sap were mixed and allowed to stand at 40° C. and pH 5·6 for 14 days. The mixture was then analysed with the aid of the three yeast species *Torula monosa*, *Saccharomyces uvarum* and *S. cerevisiae*. Found: percentage of monose 0·45, percentage of maltose negligible, percentage of maltose plus dextrins fermentable by *S. cerevisiae* (calculated as maltose) 0·36. This means that 25·6% of the theoretical maltose has been formed. After a 2 hr. action of saliva the numbers for maltose, and maltose plus fermentable dextrin are 1·23 and 2·46% respectively. This means that, if the fermentable dextrins are calculated as maltose, 77·6% of the theoretical maltose has been formed.

Action of the saps on amylopectin. As an example we give the experiment in which birch sap was allowed to act on an equal volume of a 10% amylopectin solution, without buffer, at 50° C. After 1 month it was found, by using fermentation methods, that the monose concentration of the mixture had increased by 0·405% and the maltose concentration by 0·58%, which means a yield of less than 20% of the starch originally present.

Influence of low pH *and high temperature on the saps*

(*a*) *Low* pH. 10 ml. of sap was mixed with 1 ml. of acetate buffer (pH 3·6) and allowed to stand at room temperature for 25 min. After this, 2 ml. of acetate buffer (pH 5·6) and 10 ml. of starch solution (about 4%) were added and the mixture was incubated at 34° C. for 1 day, with addition of toluene. In a parallel experiment, 1 ml. of one buffer (pH 3·6) was mixed with 2 ml. of the other buffer (pH 5·6), 10 ml. of the starch solution and 10 ml. of the sap, in the order mentioned. This mixture was also allowed to stand at 34° C. for a day. A third, similar mixture was boiled immediately after it had been prepared. To determine the reducing power of the mixtures, 1 ml. of each was mixed with 50 ml. of water and 5 ml. of the fluids obtained were treated with the Harding & Downs' reagent. In one of the experiments on birch sap we found the following differences in reducing power from the boiled blank:

Sap at low pH	0·077 mg. maltose
Sap at higher pH	0·31 mg. maltose

The difference in iodine reaction were also very illuminating. From the boiled blank a blue tinge was obtained, from the 'low pH mixture' also a blue colour, and from the 'ordinary mixture' a red-violet.

(*b*) *High temperature.* In these experiments 10 ml. of starch solution (4% for birch sap, an unknown but weaker solution for maple sap) was mixed with 10 ml. of the sap and a small quantity of buffer. After 1 day's incubation at 34° C. the reducing values and iodine reactions were compared.

Sap	Reducing value (mg. maltose)	Iodine reaction
Birch sap, untreated	0·383	Reddish violet
Birch sap, 10 min. at 67° C.	0·184	Reddish violet
Birch sap, 10 min. at 62° C.	0·310	Reddish violet
Maple sap, untreated	0·225	Reddish violet
Maple sap, 10 min. at 65° C.	0·210	Reddish violet
Maple sap, 10 min. at 60° C.	0·210	Reddish violet

So the birch sap, after the heating, still retained about half its amylolytic power (if measured in the way indicated), and the maple sap even more. This does not agree with the results of the Wijsman test which lead to the expectation that the maple sap might be richer in the less temperature-resistant enzyme. This may, however, arise from variability in the composition of the natural saps.

Comparison of maple sap with saliva and β-amylase. In order to exclude the effect of cane sugar present in the maple sap, both the saliva and the pure β-amylase (from pearl barley by a method due to Holmbergh) were diluted in sucrose solutions so that the concentration of sucrose was the same in all experiments. The maple sap had been slightly concentrated by freezing. Preliminary experiments showed that saliva diluted to one-thousandth could destroy the iodine reaction of a starch solution in about the same time as this concentrated sap, and therefore this saliva concentration was used. As a substrate we used a 2% solution of soluble starch and a *c.* 3% solution of amylopectin in the sense of Haworth *et al.* (1946). 50 ml. of substrate was mixed with 50 ml. of enzyme solution, and 1 ml. was used for every determination of the reducing power. The results are shown in Table 1.

Table 1. *Reducing power in mg. of maltose, after the action of comparable amounts of maple sap, β-amylase and saliva on starch and amylopectin*

Substrate	Soluble starch				Amylopectin			
	Time (min.)				Time (min.)			
Enzyme	105	195	300	1290	105	195	300	1290
Maple sap	0·46	0·78	1·22	4·83	0·18	0·44	0·77	3·60
β-Amylase	0·15	0·31	0·38	0·84	—	—	0·07	0·14
Saliva	0·23	0·45	0·77	1·15	0·54	0·96	1·33	2·60

It seems that saliva has a stronger effect than maple sap on amylopectin; for soluble starch the reverse is true. As no maltase and invertase activity can be demonstrated for maple sap, this may mean that β-amylase (attacking unbranched chains) is present. Another indication of this is that in the maple-sap experiment a higher reducing power was found than in that with saliva, at a time when the iodine reaction in the first case had not yet been so completely destroyed as in the latter. The action of the saliva is not continued for as long a time as the action of the maple sap.

Osazone formation. Only one representative experiment will be described. A mixture of 2 l. of 3% soluble starch and 2 l. of birch sap was allowed to stand for 3 weeks at 40° C. at a pH believed to be favourable for cellobiose formation. It was then rapidly heated and boiled for a short time. After cooling, it was evaporated down under reduced pressure to a volume of about 1 l. The fermentable sugars were then removed by means of a large quantity of fresh baker's yeast which was allowed to work for 20 hr. at 35° C., with occasional shaking. A small quantity of sodium bicarbonate was added in order to keep the pH at a not too low level. Control experiments showed that cellobiose in 1% concentration could be recovered for the most part after such treatment. After fermentation, alcohol was added to a concentration of *c.* 50% and the yeast was allowed to settle before filtration. The yeast-cake on the filter was stirred with 70% alcohol and again filtered. On mixing the two filtrates a cloudy precipitate, presumably dextrins, was obtained. The whole was evaporated, without filtering, under reduced pressure at 20–30° C. Addition of a small amount of water to the syrup obtained caused a slight precipitate. This was filtered off, the pH was adjusted to 6·0 with caustic soda and the volume was made up to 200 ml. The colour of the fluid was now a clear pale yellow, and its taste was yeasty and agreeable. 100 ml. were mixed with 2 g. of phenylhydrazine-hydrochloride and 2 g. of sodium acetate (no more being necessary because of the buffering of the fluid itself), and the mixture was heated in a boiling water-bath for 1 hr. A small amount of blackish impurity had to be filtered off rapidly at a temperature exceeding 95° C., and the filtrate was then clear and orange-brown. Between 95 and 72° C. a good deal of a voluminous light yellow precipitate was thrown down. Under the microscope it turned out to consist for the most part of small crystals of the size and shape of rod-shaped bacteria and cohering to form star-shaped clusters: the amount of echinoid crystals was very small. At a temperature below 72° C. the fluid, which was allowed to cool down very gradually, became turbid. On the next day the precipitate was collected on a filter-paper, dried and weighed. The clear fluid was also passed through another filter-paper to obtain a correction for the sodium acetate present in the precipitate. The 2·45 g. of osazone found represents 4·9 g. from the original 60 g. of starch, or not more than one-tenth of the cellobiose expected on the basis of a transformation of one-third of the starch, always assuming that osazone formation has been quantitative.

Action of emulsin on the fluid obtained after hydrolysis of starch solutions by birch sap. Part of the 200 ml. of yellow fluid obtained in the previous experiments was used to investigate cellobiase (emulsin) action. Four different mixtures were prepared, a few drops of toluene were added to each, and they were allowed to stand at a temperature of 45° C. After 2 days a known quantity of glucose was added to samples from each mixture and analyses were carried out by the micro-fermentation method. The results were as follows:

(1) Mixture of 10 ml. water, 250 mg. cellobiose and a small amount of emulsin: the cellobiose was completely hydrolysed in 2 days.

(2) Mixture of 10 ml. of fluid and a small amount of emulsin, boiled: 0·17% fermentable monoses found in the original yellow fluid.

(3) Mixture of 10 ml. of fluid and a small amount of emulsin: the emulsin led to a very small increase in monoses of 0·10–0·18%.

(4) Mixture of 10 ml. of fluid, 250 mg. cellobiose and a small amount of emulsin: all the cellobiose was recovered as monoses, showing that the fluid is not unsuitable for cellobiase action.

The maximum amount of cellobiose formed from 60 g. of starch is thus found to be less than 0·20–0·36 g. It is very likely, moreover, that this increase in monoses found after emulsin action is due to the slow hydrolysis of dextrins present in the yellow fluid. In order to investigate this possibility, the remainder of (3) was allowed to stand for another fortnight, it was then again analysed and found to contain 0·27% of monoses. This indeed means a slow increase which is the more significant since (1) and (4) show the action of the emulsin on cellobiose to be a very rapid one. Finally, another indication for the presence of dextrins in the yellow fluid is the considerable difference always found between the amounts of carbon-dioxide developed by *Torula monosa* and by *Saccharomyces cerevisiae.* This difference corresponds to more than 2% of sugar (calculated as biose). The difference between *Torula monosa* and *Saccharomyces uvarum* is much smaller.

Inhibiting action of added sugars. As an example we shall give the results of only two experiments:

(1) An alcohol precipitate from maple sap was allowed to act on a starch solution at 40° C. and pH 6·2. In test-tube experiments we found for the iodine colour after 140 min.:

No sugar	Yellow (colour of iodine)	*Maltose*	*Reddish violet*
Glucose	Yellow	Trehalose	Yellow
Fructose	Yellow	Cellobiose	Yellow
Sucrose	Brownish yellow		

(2) Fresh birch sap was allowed to work upon a starch solution at 8° C. and the pH of the sap (no buffer added). For the iodine colour after 3 hr. we found:

No sugar	Reddish brown	Sucrose	Red
Glucose	Red	*Maltose*	*Blue-violet*
Fructose	Wine red	Trehalose	Red

In all experiments maltose was found to be the only sugar exerting an inhibitory influence. This may be due to the presence of either ordinary amylases, or of starch-phosphorylase; for the plate-test, described on p. 128, when carried out under addition of maltose, reveals the fact that this sugar also has a strong inhibitory influence on starch synthesis. Though in the tree-saps we consider starch breakdown and not starch synthesis, it seemed worth while to consider the part possibly played by phosphates in the saps, and to investigate the influence of sodium fluoride, a substance known to exert strong influence on phosphorylations. Sodium fluoride was found to slow down the conversion of starch by the saps, but the influence was not stronger than that exerted on ordinary amylases such as saliva and malt-amylase. No positive influence of phosphate on the breakdown could be observed with certainty. Attempts to concentrate the phosphorylase possibly present in the saps with the aid of cadmium chloride failed. In root extracts, however, this enzyme could be demonstrated.

Adsorption experiments with alumina Cγ (see Luers & Sellner, 1925). Since our efforts to concentrate the amylases in the saps by means of ammonium sulphate and tannin precipitation failed, we tried the hydroxide mentioned above. 10 ml. of maple or birch sap, somewhat concentrated by freezing, and brought to pH 5·2 by addition of some buffer, was mixed with 10 ml. of a thick suspension of the adsorbent. After standing in the cold for some time, the fluid was centrifuged, and the supernatant used to test the enzymic activity. As a control we used the mixture of 10 ml. of the concentrated sap with 10 ml. of water. In both cases the pH was adjusted to the same value by means of a new addition of buffer. It was found that the sap treated with the adsorbent was almost unable to destroy the iodine coloration of a weak starch solution, whereas the control did so rapidly. Elution of the absorbed enzyme with phosphate buffer pH 7·5 gave a fluid with weak amylolytic activity. The results did not encourage us to try to obtain large quantities of the enzymes purified in this way.

Chemical analysis of the saps. For a full report I must refer to my thesis (Meeuse, 1943). In maple saps, sucrose is practically the only sugar present. By applying the method of Zerban & Sattler (1938), in combination with fermentation experiments, and by preparing synthetic mixtures of sucrose, glucose and cellobiose, we came to the conclusion that in some of the cases investigated by us, no reducing biose at all is present. In other cases, some may be present, but the amount is negligible.

In 1947, a comparison was made between morning and evening samples of maple sap. The amounts of sucrose found were:

Morning saps	2·62	2·33	2·51	2·26	2·29	2·47	2·12
Evening saps	2·43	2·30	2·27	2·16	2·30	2·25	2·24

On the whole, the morning saps are richer, but the difference is hardly significant.

In the various species of birch tested, as well as in *Carpinus* and *Ostrya*, we found a mixture of fructose and glucose to be present in the sap, fructose being the more important of the two. Roe's method (1934), applied to the fresh juice, gives a beautiful result after 2 min. heating. The concentration of the hexoses is much smaller than that of sucrose in maple, 1% being a high value. For a sample of birch juice we found, by applying Sieben's method: sucrose negligible, glucose 0·22%, fructose 0·57%.

Kruisheer's method, on this same juice, gave: fructose 0·575%, glucose 0·23%; when repeated, fructose 0·53%, glucose 0·24%. Application of the Barfoed principle indicated the absence of reducing bioses. *Carpinus* gave a variation in sugar concentration of the saps of 0·45–1·01%. *Ostrya* gave very low values, probably owing to contamination with rain water.

DISCUSSION

As mentioned above, Bois & Nadeau (1938*a*, *b*) were able to give an explanation of the facts reported by them which, at least at first sight, seems satisfactory. To account for the fact that maple sap flows at so low a temperature they could point to their sucrogène-amylase, which is said to possess a low-temperature optimum. The presence of cellobiogène-amylase would help in the formation of new cell-wall material (cellulose). If the glucogène-amylase in birch sap is also taken into account, all the sugars (cellobiose, glucose and sucrose) found by them in the saps can be accounted for. It is, however, most remarkable that the presence of fructose in birch sap is not mentioned. I found this sugar in all my sap analyses of birch, hornbeam and *Ostrya*, usually in quantities exceeding those of glucose. In the opinion of Lepeschkin (1927), indeed, fructose is the only sugar present in birch sap. Since I found, moreover, that both in maple and birch saps there is an amylase giving rise to products (dextrins, maltose) other than those present in the saps, doubts arise as to the justification for attempting to explain the presence of the observed sugars by the enzymes found in the saps. It is much more probable that the saps contain only a very poor remnant of the enzymes which did their work in the living cells of the root. These chemical studies are insufficient, then, to explain the phenomena of sap flow. Although an excellent beginning has been made by Burström & Krogh (1946, 1947) data of a more physiological kind are unfortunately scanty. Lepeschkin (1927) believes that in *Betula alba* and *Acer tataricum* the stimulus for flow is given by a saturation of the soil with water originating from the melting snow-cover. It may sometimes be observed that the soil temperature remains fairly constant (apart from day and night fluctuations) over the period when sap flow starts. The degree of saturation of the soil, however, steadily increases and sap flow begins when it reaches about 100%. The water is thought to dissolve the salts present in the soil, which are subsequently taken up by the root and there favour the breakdown of starch in the living cells. This was shown by Iljin (1922, 1924) to be the case in, e.g., leaf cells.

One of the most striking features of the sap flow in maple is the almost magical effect of severe cold stimuli (night frosts). Sometimes a tree which has stopped bleeding altogether, because of mild weather, starts anew, and more strongly than ever before, after a severe night frost (cf. Fig. 1). That the cold shock causes active formation of sucrose is shown by the increased percentage of sugar in the sap. Apart from these long-term fluctuations in sap production under the influence of the weather, there is a daily periodicity. Maples usually do not bleed during the night or the flow proceeds only very slowly. As this periodicity remains even when the stem is cut off immediately above the roots, it must be based on some process in the roots themselves. The sap collected in the morning has a higher sucrose content than that obtained towards the evening (p. 137). There is thus some similarity to the already described renewal of bleeding after a cold stimulus, and the processes in maple roots may be compared with those taking place in potatoes sweetened by low temperatures, also largely a matter of sucrose formation (see Müller-Thurgau, 1882, 1885*a*, *b*). In maples, then, the cooling down of the soil at night is responsible for the production of sap on the next day, while the higher temperature during daytime would explain the slowing down of starch breakdown and of bleeding, which may even cease towards the evening. On the other hand, White (1938) found fluctuations in the root pressure of tomato roots in pure culture, where the influence of changes in temperature certainly cannot be strong. According to Lepeschkin, in birch, where no sucrose is found in the sap, a decrease in temperature has a deleterious effect on sugar and sap production, and no rise in concentration can be detected.

As to the biological significance of the bleeding, the view of Bois & Nadeau that the cellobiose produced would play a part in the formation of new cell-wall material is not supported by Lepeschkin's observation that the sap flow fails to reach the uppermost buds. These buds probably contain sufficient stored food to allow sprouting, since in autumn-felled birches they sprout at the same moment in spring as in standing trees. The explanation given by Bois & Nadeau of the presence of sucrose as a result of the action of an enzyme with a low-temperature optimum certainly has no general validity. The well-known phenomenon of the bleeding of tropical palms (*Arenga*, *Borassus*, *Cocos*, *Corypha*) is relevant in this connexion: the amount of sugar produced is so high that, as with maple, it is collected on a commercial scale; and, as with maple, sucrose is the only sugar present in the sap, as shown by Tammes (1933), amongst others.

The fact that in maple as well as in palms and many kinds of nectar sucrose is the only sugar found, must be the result of special permeability relations. In general, the retention of sucrose in living plant cells seems to be rather low: as soon as a certain threshold concentration is exceeded, excretion follows. When, therefore, starch breakdown is followed by the appearance only of sucrose in a tree sap, it cannot justifiably be concluded that no other sugars were formed from the starch: the observed facts might arise from the smaller capacity of the living cells involved to retain the sucrose as compared with other sugars. Through excretion of the sucrose the chain of reactions

$$\text{starch} \rightarrow \ldots \rightarrow \ldots \rightarrow \ldots \rightarrow \text{sucrose}$$

will proceed until all the starch has been replaced by sucrose.

It is doubtful whether, in the cases cited, extraction of the living cells would ever yield the enzymes responsible for sucrose formation in sufficient quantity. In order finally to isolate these it would seem wiser to study whole complexes of living cells, selecting materials which give the largest and most rapid responses to such sudden changes as cutting off parts or chilling. The course of respiration should be studied as well as carrying out experiments of the kind described in this paper. It is possible that plant cells which already contain naturally considerable amounts of sucrose as well as starch, like those of sweet-potato tubers or root tubers of *Ranunculus ficaria*, will provide a solution of the problem of sap flow.

SUMMARY

1. No enzyme breaking down starch directly to sucrose was found in the tree saps studied.

2. A production of glucose can easily be demonstrated under physiological conditions in mixtures of birch sap with starch.

3. In maple and birch saps no formation of cellobiose from starch can be demonstrated beyond doubt.

4. Nor does birch sap contain a cellobiase which might be responsible for the presence of the glucose; but maltase is present.

5. Since maltase commonly accompanies the amylases which give rise to its substrate maltose, these latter enzymes were sought and were found in the tree saps investigated. Although difficulties were encountered owing to the weak amylase activity of the saps (that of maple sap being only one five-thousandth of that of human saliva), the presence of a mixture of relatively much α-amylase with little β-amylase in both maple and birch saps can safely be claimed on the following grounds:

(*a*) Wijsman's plate test, carried out with drops of sap concentrated by freezing, reveals the presence of two enzymes. The colourless centre is relatively large. This test alone can, of course, only differentiate the enzymes and yields no information as to their real nature.

(*b*) If, however, the saps are kept at a low pH for some time (pH 3·5), there is a marked destructive effect on the amylolytic agent, as there is on α-amylase (malt-amylase). The resistance to high temperatures is rather strong. If it is, perhaps, not as great as might be expected on the assumption that the saps contain mainly α-amylase, this may probably be due to the high dilution of the enzyme leading to extreme vulnerability.

(*c*) Soluble starch, acted upon by the saps, loses its colour reaction with iodine. At the achroic point fermentation tests show that, though the amount of free maltose is small, its presence is unmistakable. If salivary amylase is allowed to act at this stage, the quantity of free maltose rapidly increases to an amount equivalent to about 80% of the starch. This indicates that, at the achroic point, non-colouring dextrins were present besides the free maltose, but no unfermentable sugars such as cellobiose, for the latter is not broken down by saliva. It follows that there can be no room amongst the products of starch breakdown for an appreciable amount of cellobiose.

(*d*) Comparison in parallel experiments of the increase in reducing power when maple or birch sap, pure β-amylase, and α- (salivary) amylase act on starch, shows the sap to occupy an intermediate position between the two amylases. A sap which destroys the iodine reaction of a starch solution in the same time as a dilute saliva solution, gives rise to more reducing substances: in other words, the sap probably contains an admixture of β-amylase.

(*e*) The production of maltose by the sap enzymes can also be demonstrated by means of experiments with light-emitting bacteria and by the production of maltosazone.

(*f*) Of the various sugars tested, only maltose exerted a strong inhibitory action on the breakdown of starch by the sap enzymes. This can be explained by assuming either that maltose is the sugar formed, or that maltose is chemically related to the substrate of the enzymes (starch) and therefore competes with it. The second alternative involves the view that maltose is a structural unit in the starch molecule, and so conflicts with an implication of the views of Boiş & Nadeau.

(*g*) The temperature-activity and pH-activity curves are well in agreement with the assumptions made above.

(*h*) Alumina-Cγ adsorbs the amylolytic enzymes of the saps as it does the better-known amylases, e.g. from malt.

REFERENCES

BOIS, E. & CHUBB, W. O. (1942). The sap of the Birch Tree, *Betula papyrifera* Marsh. I. The Amylase System. *Canad. J. Res.* B, **20**, 114.

BOIS, E. & NADEAU, A. (1938*a*). Contributions à l'étude d'*Acer saccharum*. II. La présence d'amylases dans la sève d'érable et les produits d'hydrolyse. *Canad. J. Res.* B, **16**, 114.

BOIS, E. & NADEAU, A. (1938*b*). III. Action des amylases de la sève d'érable. *Canad. J. Res.* B, **16**, **121**.

BOIS, E. & SAVARY, A. (1942). Les glucidases et les glucides d'*Ipomoea Batatas* et de *Solanum tuberosum*. *Canad. J. Res.* B, **20**, 195.

BOIS, E. & SAVARY, A. (1945). Les amylases des micro-organismes. *Canad. J. Res.* B, **23**, 208.

BRYAN, A. H., HUBBARD, W. F. & SHERWOOD, S. F. (1937). Production of maple sirup and sugar. *Fmrs' Bull. U.S. Dep. Agric.*, no. 1366.

BURSTRÖM, H. & KROGH, A. (1946). The biochemistry of the development of buds in trees and the bleeding sap. *K. danske vidensk. Selsk., Biol. Med.* **20**, no. 2.

BURSTRÖM, H. & KROGH, A. (1947). Bleeding and bud development in *Carpinus*. *Svensk. bot. Tidskr.* **41**, 17.

FRIESE, H. & HESS, K. (1926). Ueber die Acetolyse der Cellulose. I. *Liebigs Ann.* **450**, 40.

FRIESE, H. & HESS, K. (1927). Ueber die Acetolyse der Cellulose. II. *Liebigs Ann.* **456**, 38.

GRASSMANN, W. (1928). *Neue Methoden und Ergebnisse der Fermentforschung.* München.

HALLER, R. (1945). Beiträge zur Kenntnis des Aufbaus der Starkekörner. *Helv. chim. Acta*, **28**, 450.

HANES, CH. S. (1937). The action of amylases in relation to the structure of starch and its metabolism in the plant. *New Phytol.* **36**, 101, 189.

HANES, CH. S. (1940). The reversible formation of starch from glucose-1-phosphate catalysed by potato phosphorylase. *Proc. Roy. Soc.* B, **129**, 174.

HARDING, V. J. & DOWNS, C. E. (1933). Notes on a Shaffer-Somogyi copper reagent. *J. biol. Chem.* **101**, 487.

HASSID, W. Z., DOUDOROFF, M. & BARKER, H. A. (1944). Enzymatically synthesized crystalline sucrose. *J. Amer. chem. Soc.* **66**, 1416. (See also: Doudoroff, M., Kaplan, N. & Hassid, W. Z. (1943). Phosphorolysis and synthesis of sucrose with a bacterial preparation. *J. biol. Chem.* **148**, 67).

HASSID, W. Z., MCCREADY, R. M. & ROSENFELS, R. S. (1940). Determination of starch in plants. *Industr. Engng Chem.* (Anal. ed.), **12**, 142.

HAWORTH, W. N., PEAT, S. & SAGROTT, P. E. (1946). A new method for the separation of the amylose and amylopectin components of starch. *Nature, Lond.*, **157**, 19.

HESTRIN, S., ASCHNER, M. & MAGER, J. (1947). Synthesis of cellulose by resting cells of *Acetobacter xylinum*. *Nature, Lond.*, **159**, 64.

ILJIN, W. S. (1922). Wirkung der Kationen von Salzen auf den Zerfall und die Bildung von Stärke in der Pflanze. I. Mitteilung. *Biochem. Z.* **132**, 494.

ILJIN, W. S. (1924). Abbau der Stärke durch Salze. *Biochem. Z.* **145**, 14.

KLUYVER, A. J. (1914). Biochemische Suikerbepalingen. Diss. Delft.

LEHMANN, O. (1931). Die quantitative Erfassung kleinster Mengen biologisch wichtiger Zuckerarten unter Ausschlusz reduzierender nicht-kohlenhydratartiger Körper. *Planta*, **13**, 575.

LEPESCHKIN, W. W. (1927). Untersuchungen über das Frühlingsbluten der Birke und des Ahorns. *Planta*, **4**, 113.

LUERS, H. & SELLNER, E. (1925). Beiträge zur Reinigung der Malzamylase. *Wschr. Brau.* **42**, 97.

LUTSENBURG MAAS, H. J. VAN & ITERSON JR., G. VAN (1915). A microsaccharimeter. *Proc. Kon. Akad. Wet. Amsterdam*, **18**, 258.

MEEUSE, B. J. D. (1943). Oriënterende onderzoekingen over de vorming van rietsuiker uit zetmeel in planten bij lage temperatuur. Diss. Delft.

MEULEN, H. TER (no date). *Onderzoek naar den aard van de suiker*, etc. Rotterdam: W. J. van Hengel.

MÜLLER-THURGAU, H. (1882). Ueber Zuckeranhäufung in Pflanzenteilen infolge niederer Temperatur. *Landw. Jb.* **11**, 751.

MÜLLER-THURGAU, H. (1885*a*). Beitrag zur Erklärung der Ruheperiode der Pflanzen. *Landw. Jb.* **14**, 851.

MÜLLER-THURGAU, H. (1885*b*). Ueber die Natur des in suessen Kartoffeln sich vorfindenden Zuckers. *Landw. Jb.* **14**, 909.

PLANK, J. E. VAN DER (1936). The estimation of sugars in the leaf of the mangold (*Beta vulgaris*) I–IV. *Biochem. J.* **30**, 457.

ROE, J. (1934). A colorimetric method for the detemination of fructose in blood and urine. *J. biol. Chem.* **107**, 15.

SMITS VAN WAESBERGHE, F. A. M. J. (1941). Onderzoekingen over microbenamylasen. Diss. Delft.

SPENCER, C. C. (1929). Die Acetolyse der Baumwollecellulose. *Cellulose-Chem.* **10**, 61.

TAMMES, P. M. L. (1933). Observations on the bleeding of palm trees. *Rec. Trav. bot. néerl.* **30**, 514.

WEBER, A. PH. (1941). *Over de werking van de zetmeelsplitsende enzymen uit gerst en mout.* Publ. L. E. B.-fonds, Wageningen.

WHITE, P. R. (1938). 'Root pressure'—an unappreciated force in sap movement. *Amer. J. Bot.* **25**, 223.

WIJSMAN, H. P. (1889*a*). De diastase, beschouwd als mengsel van maltase en dextrinase. Diss. Amsterdam.

WIJSMAN, H. P. (1889*b*). La diastase considérée comme un mélange de maltase et dextrinase. *Rec. Trav. chim. Pays-Bas*, **9**, 1.

ZERBAN, F. W. & SATTLER, L. (1938). Analysis of sugar mixtures, containing dextrose, levulose, maltose and lactose. *Industr. Engng Chem.* (Anal. ed.), **10**, 669.

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DISTRIBUTION OF ACETYLCHOLINE AND HISTAMINE IN NETTLE PLANTS

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In a previous paper we have described the occurrence of acetylcholine, histamine and a third unidentified smooth muscle-contracting substance in the hair fluid of *Urtica urens*, the annual nettle. The vascular reactions produced in the human skin and the sensations elicited by the sting of the nettle hair could be accounted for by the combined effect of acetylcholine and histamine. Both substances were also found in the leaf tissue (Emmelin & Feldberg, 1947).

In the experiments described in the present paper it could be shown that acetylcholine and histamine occur also in high concentrations in the hair fluid of the perennial nettle, *U. dioica*. No experiments have been carried out on the occurrence of the third unidentified smooth muscle-contracting substance, but the distribution of histamine and acetylcholine in the annual and perennial plant have been examined in more detail. Since the publication of our previous experiments our attention has been drawn to a paper by Starkenstein & Wasserstrom (1933) who had found that extracts of whole nettle plants caused contractions of smooth muscles, a fall in arterial blood pressure and inhibition of the heart. No indication was given as to the nature of the substance or substances responsible for these effects. In the light of the experiments presented in this paper, histamine and acetylcholine were probably the active principles, or at least the main active principles in their extracts.

Methods

The procedure adopted was the same as that described previously. For the routine assay of acetylcholine the eserinized frog rectus muscle was used. For the identification the following tests were applied: sensitivity to alkali and to cholinesterase, stability to boiling in acid solution, the stimulating effect on the isolated guinea-pig's intestine, the abolition of the effect by atropine but its resistance to benadryl. For the routine assay of histamine the atropinized guinea-pig's intestine preparation was used; for the identification use was made of the antagonistic action of neoantergan. All extracts were made with saline or acidified saline. The completeness of the extraction was controlled by showing that re-extraction of the insoluble material with acid saline and boiling yielded no active substance or traces only of histamine and acetylcholine. The values for histamine refer to the dihydrochloride, those for acetylcholine to the chloride.

Results

Hairs. The fluid from the hairs of *U. dioica* contains acetylcholine as well as histamine (Table 1). The acetylcholine content per individual hair was usually between 0·04 and 0·07 γ, but in one experiment with particularly small hairs the value was 0·013 γ. There

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was no difference between the content of acetylcholine in the hairs from the main stems and from the leaves. In corresponding experiments with *U. urens* the hairs from the stem had been found usually to contain a larger amount of acetylcholine than those of the leaves. This difference was attributed to the larger size of the hairs situated on the stems. In *U. dioica* there is no obvious difference in the size of the hairs situated on the stems or on the leaves. The histamine content was greater in the hairs from the stem than from the leaves. Similar results had been obtained in the experiments with *U. urens*.

Table 1. *Acetylcholine and histamine content of hairs from* Urtica dioica

Origin of hairs	No. of hairs used for assay	Acetylcholine in γ per hair	Histamine in γ per hair
Stem	30	0·067	—
Leaves	30	0·046	—
Stem	500	0·040	0·01
Leaves	500	0·049	0·0035
Stem (small hairs)	100	0·013	—

Leaves. In order to obtain correct values it is necessary to remove as carefully as possible the hairs before extracting the leaf tissue, otherwise the values obtained are too high. For instance, two samples of leaf tissue from adjacent leaves of *U. dioica* were prepared, care being taken that the larger veins of the leaves were not included in the samples. In the one sample the hairs were left on the leaf tissue and included in the extraction; the acetylcholine content was 0·7 γ/10 mg. tissue. In the other sample, in which the hairs had been removed before extraction, the value was 0·095 γ/10 mg. tissue only. The corresponding values for histamine in the two samples were 0·3 and 0·17 γ respectively. As long as the hairs remained on the leaf tissue the values obtained for acetylcholine were higher than those for histamine, but when the hairs had been removed the order was more often reversed. It is possible that incomplete removal of the hairs was the reason why this reversal did not always happen. The histamine and acetylcholine extracted from leaf tissue is not derived, or derived specially, from those small spots from which the hairs originate, i.e. the bases of the hairs, because the results were the same if the small areas of tissues used for extraction were carefully cut out between the hairs. The results for both *U. urens* and *dioica* are given in Table 2. When both acetylcholine and histamine were assayed in the same sample the two values are given below each other in the table. It will be seen that there are great individual variations, but that apart from these the histamine concentration is higher in the leaf tissue of *U. urens* than of *U. dioica*.

Table 2. *Acetylcholine and histamine in* γ *per* 10 *mg. leaf tissue (hairs removed)*

U. urens:									
Acetylcholine	0·09	0·14	0·05	0·02	0·27	0·18	—	—	—
Histamine	1·0	1·0	0·83	—	—	0·19	0·5	—	—
U. dioica:									
Acetylcholine	0·095	0·095	0·67	0·005	0·35	0·12	0·21	0·3	0·65
Histamine	0·2	0·17	0·2	0·17	0·063	—	—	—	—

Stems. When the hairs had been removed carefully the values for acetylcholine varied between less than 0·1 and more than 1·0 γ/10 mg. tissue; those for histamine between 0·017 and 0·7 γ/10 mg. tissue. The two substances were not found to be uniformly distributed in the stem tissue. When the greenish or greenish reddish chlorophyll-containing outer layer, the assimilating cortex, was peeled off and examined separately from the stele, it was found to contain both substances in higher concentration (Table 3). The concentration of acetylcholine in the assimilating cortex is on the average higher than that found in the leaf tissue. The increase is particularly pronounced in *U. urens*. The histamine concentration in the assimilating cortex of the stems of *U. dioica*, on the other hand, is certainly lower than that of the leaf tissue, whereas in *U. urens* the concentration in these two tissues is practically the same.

Roots and rhizomes. No histamine could be detected in any part of the roots and rhizomes of *U. dioica*, whereas it occurred in relatively high concentrations in the roots of *U. urens*. Acetylcholine was present in the roots of both plants, but in much lower concentration in *U. urens* than in *U. dioica*.

Table 3. *Distribution of acetylcholine and histamine in outer and inner layer of stem*

Content in γ per 10 mg. tissue

Plant	Acetylcholine		Histamine	
	Assimilating cortex	Stele	Assimilating cortex	Stele
U. urens	0·45	0·06	1·1	0·23
U. urens	0·5	0·06	0·8	0·17
U. dioica	0·26	0·11	0·03	0·013
U. dioica	0·07	0·02	0·05	0·01
U. dioica	0·5	0·14	—	—

Table 4. *Distribution of acetylcholine and histamine in the underground tissue of* Urtica urens

Exp. no.	Acetylcholine in γ per 10 mg. tissue				Histamine in γ per 10 mg. tissue		
	Rootlets	Main root			Rootlets	Main root	
		Whole	Cortex	Stele		Cortex	Stele
1	0·55	—	0·09	0·01	1·1	0·38	0·06
2	0·19	—	0·05	—	0·55	0·7	—
3	—	—	0·24	0·06	—	2·7	0·65
4	0·14	—	0·08	—	—	1·0	—
5	0·13	0·08	—	—	—	—	—
6	0·13	0·03	—	—	—	—	—
7	—	—	0·03	0·02	—	—	—
8	—	—	0·54	0·01	—	—	—
Mean	0·23	0·06	0·17	0·025	0·8	1·2	0·36

In Table 4 is shown the distribution of acetylcholine and histamine in the underground tissue of *U. urens*. The values vary greatly in different plants, but the differences between the different parts of the roots are evident in all experiments. The mean values are given at the bottom of the table. The small rootlets contain acetylcholine in a higher concentration than the main roots. In these the concentration of acetylcholine is highest in the cortex, but even here not as high as in the rootlets. Histamine also is more concentrated in the cortex than in the central stele of the main roots, but there is no constant difference between rootlets and main roots. In all parts the concentration of histamine was found to be greater than that of acetylcholine. The histamine concentration of the rootlets and of the cortex of the main root was of the same order as that observed in leaf tissue and in the assimilating cortex of the stems.

No histamine could be found in any part of the underground tissue of *U. dioica*, although the sensitivity of the assay was such that amounts of histamine greater than 0·005 γ/10 mg. tissue would have been detected. The acetylcholine was present in the rootlets, roots and rhizomes. The rootlets contained between 0·45 and 1·9 γ acetylcholine in 10 mg. tissue, usually the finest hairlets giving the lowest values. The rhizomes contained, on the other hand, as much as 3–7 γ of acetylcholine in 10 mg. tissue. The greatest proportion of this acetylcholine is derived from the brownish cortex and the adjacent greenish phloem. In these between 5·6 and 135 γ (average 37 γ) per 10 mg. tissue were found, an average concentration of acetylcholine of about 1 : 250, and in one instance even of about 1 : 70.

Synthesis of acetylcholine. In a few experiments the methods described by Feldberg (1945) and by Feldberg & Mann (1945) for the synthesis of acetylcholine in animal tissue were applied to saline extracts prepared either from fresh or from acetone-dried tissue of the leaves, roots and rhizomes of the nettle plant. No synthesis occurred on incubation of these extracts for 2–3 hr. at 25 or 37° C. under aerobic or anaerobic conditions.

Table 5. *Distribution of acetylcholine in different parts of rhizomes of* Urtica dioica

Exp. no. ...	1	2	3	4	5	6	7	8
Outer part (whole)	5·6	16	22	28	135	—	—	—
Cortex	—	—	—	—	—	24	25	54
Phloem	—	—	—	—	—	24	40	—
Inner part	—	—	—	—	—	—	0·9	—
Wood	—	—	—	—	0·4	2·9	—	2·6
Pith	—	—	—	—	0·1	1·1	—	1·0

DISCUSSION

Our results show that acetylcholine as well as histamine, which previously had been found in the hair fluid of *U. urens*, occur also in the hair fluid of *U. dioica*, the perennial plant. This was to be expected, since these substances were found to be responsible or at least mainly responsible for the reactions of the human skin to the nettle sting. Both substances were found to occur not only in the hair fluid, but also in other parts of the plant. A study of their distribution revealed differences between *U. urens* and *dioica*.

In *U. urens* the concentrations of acetylcholine and of histamine appears to be of the same order in the leaves, in the outer cortex of both stem and main root, and in the rootlets. At least the variations between individual plants and between individual values obtained from the same part of different plants are so great as to render it impossible to detect, with the relatively few determinations so far made, for each tissue, significant differences. The concentration of acetylcholine in these tissues varied between 1 in 500,000 and 1 in 20,000 with a mean concentration of 1 in 50,000. The mean concentration for the separate tissues was: leaf 1 in 80,000; assimilating cortex of stem 1 in 20,000; cortex of main root 1 in 60,000 and rootlets 1 in 40,000. The concentration of histamine in these tissues varied from about 1 in 50,000 to 1 in 4000 with a mean concentration of 1 in 10,000.

The mean concentration for the separate tissues was: leaf 1 in 14,000; assimilating cortex of stem 1 in 10,000; cortex of main root 1 in 8000 and rootlets 1 in 12,000. These concentrations are much weaker than those observed in the hair fluid. The substances may therefore be transported to the hairs and there concentrated. In that case acetylcholine would have to be concentrated to a greater extent than histamine.

In *U. dioica* acetylcholine and histamine are not uniformly distributed. Despite the great individual variations found it could be shown that there are real differences in the various tissues. The mean concentration of acetylcholine in the leaves and in the assimilating cortex of the stem, i.e. the green tissues, was about equal, over 1 in 40,000, but increased to 1 in 9000 in the rootlets, to 1 in 2000 in the rhizome and in its cortex and phloem to even 1 in 250. Histamine was not found in all parts of the plant; it was absent in the rhizomes, roots and rootlets. Its concentration in the leaves was about four times that in the assimilating cortex of the stems where it occurred in a mean concentration of 1 in 250,000 only.

It was hoped to obtain from the distribution of acetylcholine and histamine in the different parts of the nettle plants some information about the possible site of the formation of these substances. The rather uniform distribution, below and above ground, in the soft tissues of *U. urens* made it impossible to obtain any indication in which special tissue these substances might be formed in the annual plant. But also the uneven distribution of these substances in the perennial plant does not allow us to state with certainty the tissue responsible for their formation. It may well be that the very high concentration of acetylcholine in the rootlets and rhizomes is not associated with its formation here, but with the fact that it has accumulated in these perennial parts during previous years. If the acetylcholine were formed below the ground it might be formed either by soil bacteria or by a tissue enzyme present in the rootlets and in the cortex and phloem of the rhizome; if formed by the action of a tissue enzyme the conditions for its activity, however, would be different from those which favour the synthesis of acetylcholine in animal tissue. From the distribution of histamine in the different parts of *U. dioica*, outside the hair fluid the most likely tissue for the site of its formation is the green tissue of the leaves, where it might be formed either by a tissue enzyme or even by ultra-violet decarboxylation of histidine.

SUMMARY

1. The presence of acetylcholine and histamine in high concentrations in the fluid of the hairs, previously found for *Urtica urens* has now also been established for *U. dioica*, the perennial plant.

2. The distribution of acetylcholine and histamine in the different tissues of the annual and perennial nettle plant has been examined.

3. In *U. urens* acetylcholine and histamine are rather uniformly distributed in the following parts: the leaves, the outer cortex of both stem and main root, and the rootlets. The concentration in these tissues varies greatly in different plants, the mean concentration is 1 in 50,000 for acetylcholine and 1 in 10,000 for histamine.

4. In *U. dioica* acetylcholine and histamine are not so uniformly distributed as in *U. urens*. The mean concentration for acetylcholine in the leaf and the assimilating cortex of the stem was over 1 in 40,000, in the rootlets 1 in 9000, in the rhizome 1 in 2000 and in its cortex and phloem even 1 in 250. Histamine was not present in the underground

tissue. It occurred in the leaves in a mean concentration of about 1 in 60,000 and in the assimilating cortex of the stem of about 1 in 250,000.

5. Acetylcholine and histamine may not be formed in the hairs but transported to them and there concentrated. The possible site and mode of formation of these substances in nettle plants is discussed.

We should like to make grateful acknowledgement to Dr K. R. Sporne of the Botany School, Cambridge, for aiding us in the anatomy of the plants.

REFERENCES

EMMELIN, N. & FELDBERG, W. (1947). *J. Physiol.* **106**, 440.
FELDBERG, W. (1945). *J. Physiol.* **103**, 367.
FELDBERG, W. & MANN, T. (1945). *J. Physiol.* **104**, 411.
STARKENSTEIN, E. & WASSERSTROM, T. (1933). *Arch. exp. Path. Pharmak.* **172**, 137.

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SUCCINATE AND PLANT RESPIRATION

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(With 14 figures in the text)

CONTENTS

		PAGE
I.	Introduction	149
II.	Materials and methods	149
III.	Respiratory drifts in distilled water	151
IV.	Preliminary work on pH effects	152
V.	The effect of succinate on oxygen uptake	154
	(1) Effects of the pH on the succinate solution	154
	(2) Concentration of succinate solutions: reversibility	156
	(3) Succinate and potassium chloride	159
	(4) Inhibition	160
VI.	The effect of succinate on the evolution of CO_2	161
	(1) Succinate addition at pH 4	162
	(*a*) Concentration and R.Q.	163
	(*b*) Reversibility	163
	(*c*) Effect of acids related to succinic	163
	(2) Succinate addition at pH greater than 4·0	165
VII.	Preliminary experiments on the uptake of succinic acid	166
VIII.	Discussion	168
IX.	Summary	170
	References	171

I. INTRODUCTION

This paper describes experiments which were begun in order to determine whether the Krebs' organic acid cycle functions in plant tissues. It was proposed to test the respiratory response of carrot-root tissue slices to many of the organic acids which are intermediates in animal carbohydrate metabolism. However, preliminary experiments showed that intact carrot-tissue slices present a much more complex picture than do breis, homogenates or isolated enzyme systems from animal tissues. We have therefore considered it worth while to report the results of a detailed study of the effect of one acid on carrot respiration.

Cytochrome oxidase has been demonstrated in carrot root tissue by Prof. D. R. Goddard (personal communication). In animal tissues this oxidase system is reversibly reduced by the succinic dehydrogenase-succinic acid system. Since a similar coupling might be expected with carrot cytochrome, succinic acid was chosen as the first organic acid to be tested. This acid has already been shown to be a stimulant of plant respiration (Bennet-Clark & Bexon, 1943; Machlis, 1944).

II. MATERIALS AND METHODS

Carrots, Danver's Half Long, variety Stock's Prolific, were topped and tipped, and the cut-ends immersed in liquid-paraffin wax. The roots were then placed on trays in a refrigerator, the temperature of which was maintained at about 5° C. This treatment preserved the carrots, with relatively little dehydration, for about 4 months. Storage of carrots, rather than the collecting of fresh roots as required, ensured that the experimental material was not such a variable factor. The roots were usually harvested and stored in March, July and November.

For use in experiments, the roots, after being thickly peeled, were cut into disks 0·80 cm. in diameter and 0·10 cm. in thickness. The sliced tissue was rinsed several times and then aerated in distilled water until required. The time of this preliminary washing varied from 1 to 300 hr. If the suspension water became cloudy, or the disks lost their brittle character, bacterial infection was suspected, and the tissue was discarded. Slices were normally taken from the secondary phloem. Secondary xylem tissue (largely parenchymatous) has larger air spaces, and, on a fresh-weight basis has a respiration rate in distilled water of a little less than two-thirds that of phloem tissue. Qualitatively, however, its respiratory behaviour is similar to that of phloem parenchyma. As shown in Table 1 the respiration rates on a protein basis are similar for xylem and phloem parenchyma.

Table 1. *Protein contents, and respiratory rates of xylem and phloem parenchyma at* 100 *hr. from cutting* (*Type A carrots*)

Tissue (parenchyma)	Dry wt./fresh wt. (%)	Protein content as % of dry wt.	O_2 uptake/mg. dry wt./hr. 25° C. (cu. mm.)	O_2 uptake/mg. protein/hr. 25° C. (cu. mm.)
Phloem	7	0·48	1·43	298
Xylem	5	0·40	1·20	300

Respiration rates were determined at 25 ± 0·1° C. by measuring the oxygen uptake of approximately 1·1 g. (25 disks) of tissue in Warburg vessels, 0·10 c.c. of 20% potash being in the centre well to absorb CO_2. At the beginning of each experiment the slices were dried lightly, weighed and transferred to the Warburg vessels with 3·60 ml. distilled water. After the steady rate in distilled water had been established, the desired solution (0·40 ml.) was tipped from the side arm. When the effects of more than one substance were being tested, the vessels were later removed, a measured volume (0·4–0·5 ml.) of solution inserted into the side arm, and, after equilibration in the constant temperature bath, tipping was repeated. A similar volume of distilled water was added simultaneously to sets of tissue which acted as controls.

In experiments with potassium cyanide, the vessels were removed for its addition, and the potash in the centre well was replaced by appropriate potassium hydroxide-potassium cyanide mixtures (Krebs, 1935). Cyanide solutions were always prepared immediately before an experiment.

When reversibility was being tested, the tissue medium was completely removed by pipette. The disks, retained in the Warburg vessel, were then rinsed four times with distilled water, and finally dried lightly by inserting a filter-paper roll. Then 4·0 ml. distilled water was pipetted into the vessel. The entire procedure occupied about 7 min. The organic compounds used were succinic, mono-methyl-succinic* glutaric, adipic and malonic acids. Solutions of these substances were made by dissolving the acids in distilled water and adjusting the pH with potassium hydroxide. The concentrations quoted refer to the final strengths in contact with the tissue.

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* Made by the method of Higginbotham & Lapworth (1922). The racemic mixture had a m.p. 109–110° C.: its purity was established by paper chromatography (Lugg & Overell, 1948).

The direct method of Warburg (Dixon, 1943) was used in measuring respiratory quotients, since this gives successive values over short time intervals. For these experiments very carefully matched disks were chosen. This method is normally suspect when the R.Q. values are high (as in many of our experiments) for only one set of each pair of tissue samples is exposed to high CO_2 concentration. Replicate measurements were therefore made, as a check, by the direct and indirect methods of Warburg and also by the Dickens-Simer method (Dixon, 1943). The three methods gave closely similar figures for R.Q. even when this was above 2·0. Turner (1938) also showed that for carrot tissue the direct method gave results agreeing with those obtained by the Dickens-Simer method over an R.Q. range of 1·0–5·0.

Respiration will be referred to as $Q^{F}_{O_2}$ which we define as the volume of oxygen in c.c. absorbed by 1 g. fresh weight of tissue per hour, at 25° g.

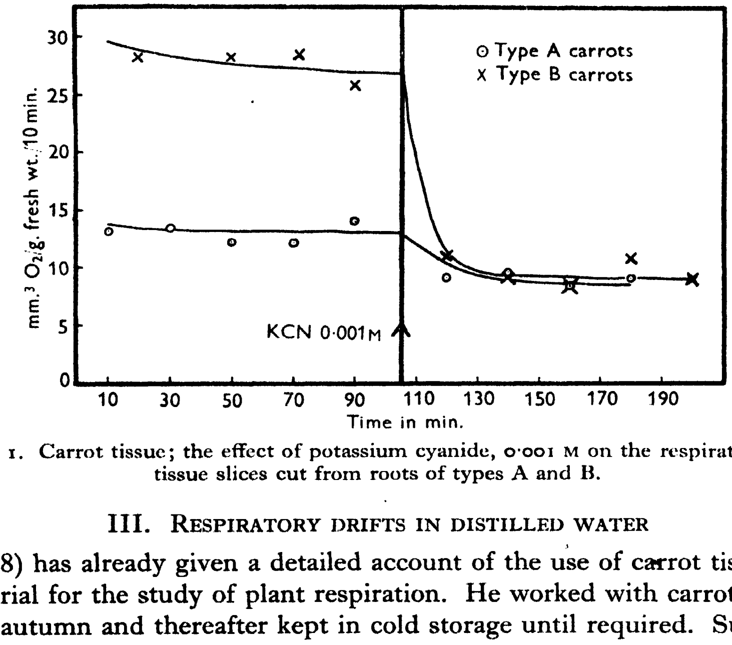

Fig. 1. Carrot tissue; the effect of potassium cyanide, 0·001 M on the respiration of tissue slices cut from roots of types A and B.

III. RESPIRATORY DRIFTS IN DISTILLED WATER

Turner (1938) has already given a detailed account of the use of carrot tissue as experimental material for the study of plant respiration. He worked with carrots harvested in the English autumn and thereafter kept in cold storage until required. Such tissue, cut into disks as outlined above and suspended in distilled water, gave respiration-rate drifts which rose sharply till about 50 hr. from cutting and then fell gradually and almost asymptotically to a low value. Thus, soon after cutting, their respiration rate was high ($Q^{F}_{O_2}$ = approx. 170) but then it drifted downwards to a $Q^{F}_{O_2}$ of about 60 after some 200 hr. (Turner, 1940).

Most of the carrots used in the present investigations gave respiration-rate time curves somewhat similar to those described above. Their typical respiratory time drift in distilled water is shown in Fig. 6, curve 3. But it will be seen that the initial rise was not pronounced (the greatest $Q^{F}_{O_2}$, obtained at 20 hr., was only 138), nor was the final steady rate as low as that recorded by Turner. However, carrots which in general conformed to this falling rate will be grouped together as type A carrots.

One batch of carrots, harvested in midwinter (July) 1946, consistently showed much higher rates in distilled water. Fig. 6, curve 2, presents a typical respiration curve for this tissue, which we shall designate as type B. That this enhanced respiration of the type B carrots was cyanide sensitive is shown by Fig. 1: application of 0·001 M-KCN always depressed the respiration of tissue slices, types A or B, to about the same level—

$$(Q^{\mathrm{F}}_{\mathrm{O}_2} = 40\text{–}60).$$

We shall define this rate of oxygen uptake, which remains steady in the presence of 0·001 M-KCN, as the 'ground respiration'.

We believe, however, that the physiological dissimilarity of carrots of types A and B is quantitative rather than qualitative, since both react to the same inhibitors (KCN and malonic acid) and stimulants (succinic acid and KCl). Hence we consider that results obtained from either type of tissue are generally applicable. Further consideration of the relation between 'wound' respiration and 'ground respiration' will be given in another paper.

IV. PRELIMINARY WORK ON pH EFFECTS

It may be stated immediately that solutions of potassium succinate, when added to carrot tissue which is respiring at a steady rate in distilled water, invariably cause a rapid increase in respiration. This typical behaviour is illustrated in Fig. 7. It has been recorded previously, but it has been overlooked that the increase (in oxygen uptake at least) due to potassium succinate is similar to that caused by solutions of inorganic salts, e.g. potassium chloride, which induce a salt respiration (see Figs. 8, 9). Both stimulants bring about a rapid rise in oxygen uptake to a new steady state, which is established within 30 min. (half-time, 10 min.). The new level of oxygen uptake is then maintained or it falls only slightly, over a period of several hours.

It is therefore necessary to establish whether an effect on respiration, due to potassium succinate, is a salt effect, associated with the 'accumulation' of the ions, or whether it is due to reactions involving the succinate ion or succinic acid molecule acting as substrates. One way of testing this is to supply the compound at different hydrogen-ion concentrations so as to alter the relative amounts of undissociated molecule and ions. It has already been shown (Turner & Hanly, 1947) that the respiratory inhibitor malonic acid does not exert its characteristic effect on the living cell unless it is applied in acid solution. It seems likely that the same may apply to other organic acids which act as respiratory stimulants. Thus, Anderson (1945) showed that the colourless alga *Prototheca* metabolized both pyruvic and lactic acids only in acid media. Myers (1947) showed that succinic, pyruvic and glycollic acids were respired by *Chlorella* in media of pH 3·8, but not 6·8. We may also refer to the well-known work of Warburg & Negelein (1920) on nitrate, and of Osterhout (1925) on hydrogen sulphide.

However, alteration of the pH of the medium brings about further complications, and it was necessary to discover something of the effect of pH *per se* on carrot respiration before results with succinate could be interpreted.

In most of our work we have suspended the carrot slices initially in distilled water, but in order to study the effect of pH a buffered solution must be used. Early work by Thomas (1930) on the effect of pH on carrot-root respiration is rendered suspect because he used, amongst others, citrate buffers; we now know that citrate can be used as a metabolite by carrot tissue (unpublished). It is impossible to use acetate buffers for the same reason, and we have found phthalate buffers to be toxic. While realizing that any buffer solution may bring about a salt effect, and that phosphate is itself important in respiration, we have been driven to investigate our problem by using phosphate solutions over a range of pH from 4·5 to 7·5. We used mixtures of potassium di-hydrogen phosphate and disodium hydrogen phosphate with a total molar concentration of 0·07 M. Table 2 shows the alteration in the ionic concentrations associated with the pH changes. The experiment, which was repeated several times, consisted in measuring the gas exchange of five sets of tissue slices, first in distilled water: one set was kept as a control and each of the others was given phosphate buffer (Fig. 2). There was consistently a marked stimulation of the oxygen uptake brought about by the less acid solutions. The buffer solution of pH 4·5 brought about no significant change from the normal. We are not prepared at this stage to attribute the rise in the less acid buffers to a pH effect *per se*, as it might be a salt effect. At pH 4·5, however, there are also high concentrations of the potassium ion and of H_2PO_4'. In order to test whether the lack of a salt effect under these conditions was due to the low pH, we added potassium chloride to all the tissues after the respiration had become steady in the buffer solution (Fig. 2). A salt effect was always obtained with the potassium chloride and it was greater in the more acid solutions.

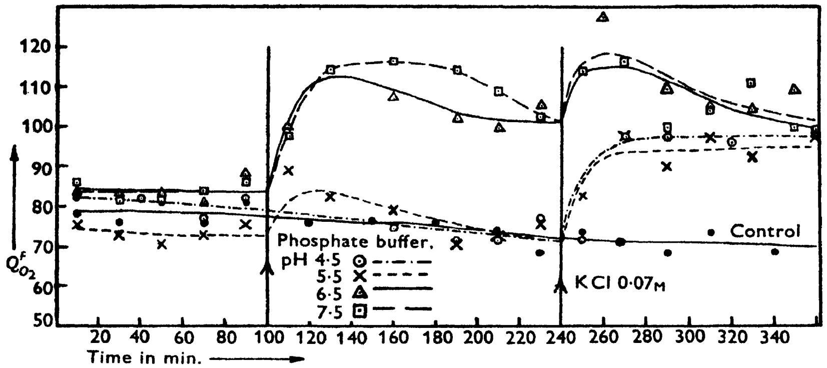

Fig. 2. Carrot tissue; oxygen uptake as affected by phosphate buffer over a range of pH. Subsequent response shown towards potassium chloride, added to the phosphate solutions. The control set of tissue was in distilled water.

Table 2. *Molal concentrations in phosphate buffer solutions of total molarity* 0·07 M.

pH	[Molecule] × 1000	$[H_2PO_4']$ × 1000	$[HPO_4'']$ × 1000	[K+] × 1000	[Na+] × 1000	Total cation molality × 1000
4·5	2·9	695	2	700	—	700
5·5	—	686	9	686	18	704
6·5	—	585	115	585	231	816
7·5	—	236	465	236	929	1165

As a possible explanation of the lack of a salt stimulation by phosphate alone at pH 4·5 we may suggest that the tissue is much less permeable to H_2PO_4' than it is to HPO_4'' (see Table 2). Petrie (1938) and Ulrich (1941) have both come to the conclusion that the monovalent phosphate ion does not penetrate cell membranes. Tissue already stimulated to give a salt respiration by HPO_4'' (at pH 5·5–7·5) would be expected to be relatively insensitive to potassium chloride. Undoubtedly, however, a great deal more work is required on this aspect of the problem, and in particular a study of the pH effect on salt respiration would be valuable. Our results emphasize the fact that the use of neutral phosphate media in respiration studies is open to criticism, since the stimulations induced by such solutions may mask other responses. However, the conclusions which are pertinent to the present investigation are that phosphate buffers at pH 4 neither inhibit nor stimulate carrot respiration; that tissue at such pH responds to the addition of neutral salts; and that solutions of higher pH may cause stimulation.

V. THE EFFECT OF SUCCINATE ON OXYGEN UPTAKE

(1) *Effects of the* pH *of the succinate solution*

We have tested the effect of succinic acid at pH values ranging from 4 to 7·6, and the results are presented graphically in Fig. 3. The rates of respiration have been expressed in terms of percentage above or below the control rates only within the bounds of one experiment. Comparison on the percentage basis between material cut at different times and from different roots is considered meaningless owing to the variation in normal respiration associated with the time from cutting and with season.

In contrast to the effect of phosphate, maximum stimulation of oxygen uptake is always induced by potassium succinate solutions of pH 4–5; solutions at higher pH, e.g. pH 7, cause much less marked stimulation. Since succinic acid is a dibasic weak acid, it exists in solution as an equilibrium mixture of the molecule and its two ions, the relative concentrations being fixed for any particular pH. Taking the value of pK_1 as 4·18 and of pK_2 as 5·60, these molecular and ionic concentrations have been calculated and are presented in Fig. 4. We also include the figures for potassium-ion concentration over the same range, the pH having been adjusted by adding potassium hydroxide to the solution of the acid. These graphs have been used to determine the molecular and ionic concentrations which will be quoted.

In the succinate solutions which cause maximum stimulation (pH 4–5), the concentration of the succinic acid molecule is from 60 to 10% of the total molarity. In solutions less acid than about pH 5·5 the relative concentration of undissociated acid is extremely low (see also Table 4); the small respiratory stimulus caused by these solutions is ascribed therefore to a salt effect.

Another factor influencing respiration rate at high pH may be the absorption by the tissue of excess cations from the succinate solutions, these inducing an 'Ulrich organic acid effect' (Ulrich, 1941). Indirect evidence that carrot disks do preferentially absorb potassium ions from neutral solutions of potassium succinate has been obtained from pH measurements. Always, by the end of an experiment, the pH of the medium, initially adjusted to pH 7, is found to be between pH 5·9 and 6·3. This change may be partly attributable to the high partial pressure of CO_2 (maintained by respiration) which would increase the concentration of HCO_3' and H^+ ions in solution. It may also be due to exchange of potassium ions with hydrogen ions. Such has been observed by Conway & Brady (1947), and by Ulrich (1941) who noted that barley roots suspended in potassium citrate solution, absorbed the cations in excess, with an attendant drop of the pH of the external medium from 7·25 to 5·76. Ulrich also found that this excess cation intake was accompanied by organic acid synthesis within the cells, the acid anion so formed acting as a buffer against the potential rise in pH of the cell sap. Simultaneously with acid formation there was a decrease in the R.Q. to about 0·8 and an increase in the rate of oxygen absorption. This metabolic change we have already referred to as an Ulrich effect. The possibility of its existence in our experiments complicates the interpretation, and we shall return to it in the section dealing with carbon dioxide output.

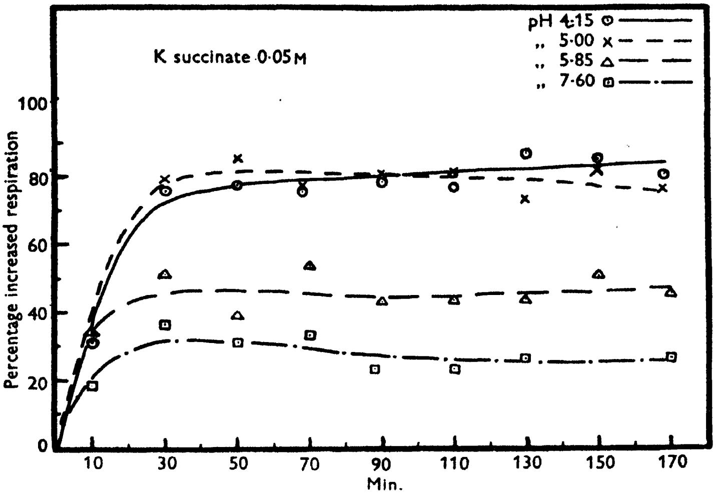

Fig. 3. Carrot tissue; stimulation of O_2 uptake from (zero), time of tipping solutions of potassium succinate, 0·05 M, adjusted to different values of pH.

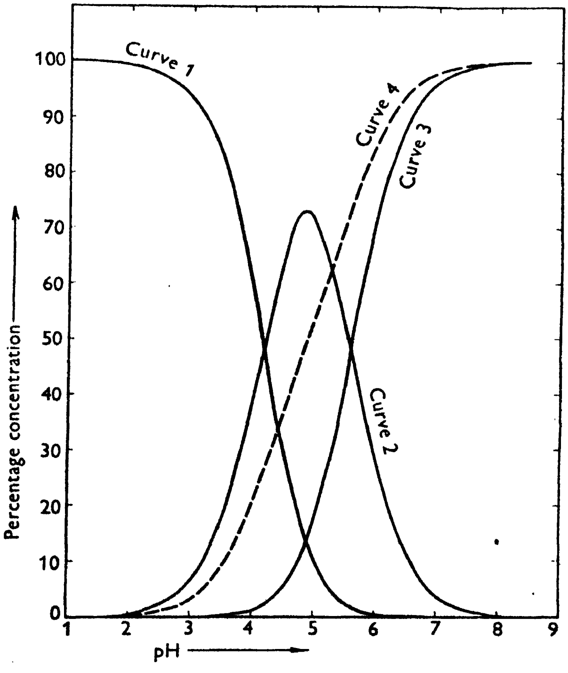

Fig. 4. Dissociation curve of succinic acid, the pH adjusted with potassium hydroxide. Curve 1, percentage concentration of undissociated acid; curve 2, percentage concentration of univalent succinate ion; curve 3, percentage concentration of divalent succinate ion; curve 4, half the percentage concentration of potassium ion. (Thus at pH 5·0 $K^+ = (50 \times 2)\%$.)

In the pH range of 4·0–5·5 the ionic concentration is still sufficient to bring about salt respiration. However, it is at this lower pH that maximum respiration rates are obtained and in which the undissociated molecule is present in the highest concentration. We may therefore ascribe part at least of the stimulation to the effect of the undissociated molecule itself, as it is well known that some organic compounds enter cells rapidly when in the molecular state.* The succinic acid molecule may exert its influence by catalysing some organic acid cycle such as the Krebs cycle, or it may act as a respiratory substrate in the same or in another system. The rest of this paper describes experiments designed to distinguish between the ionic and the molecular effects of succinate solutions, and in particular to determine the nature of the cell's reaction towards the undissociated molecule.

(2) *Concentration of succinate solutions: reversibility*

The degree of stimulation caused by succinic acid at pH 4 varied with the concentration and also with the season at which the carrots were dug and the time for which the disks were washed. The typical picture is given in Fig. 5 in which it is shown that the optimal stimulation lies within the range 0·02–0·05 M. Higher concentrations, for example, 0·10 M solutions, may cause a submaximal increase, or when applied to long-stored, well-washed tissue, they may bring about irreversible inhibition as a result of damage to the cells. Such damage may be due to the inability of cell tissue to maintain normal buffered conditions when in the presence of a concentrated external buffer solution of low pH, or to the effect of the higher concentration of the succinic acid molecule (which is lipoid soluble) on the cell membrane.†

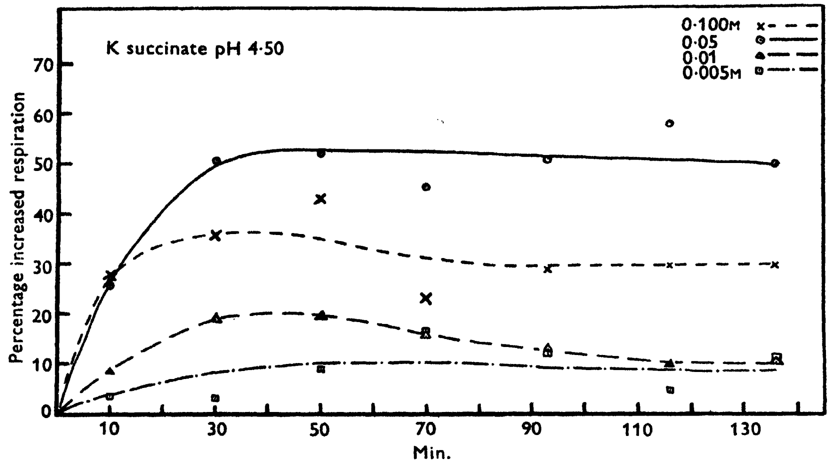

Fig. 5. Carrot tissue; percentage stimulation of O_2 uptake from (zero), time of tipping potassium succinate solutions at increasing dilutions, pH 4·50.

The response to different succinate concentrations is correlated with the initial rate of respiration, which varies with the season and with the time from cutting. Thus type A carrot disks which have been cut for 100 hr. ($Q_{O_2}^{F} = 80$) show some stimulation in the presence of 0·001 M-succinate solutions, while the stimulation with 0·005 M is pronounced. However, carrots of type B, which at 100 hr. have a much higher initial rate $(Q^{\mathrm{F}}_{\mathrm{O}_2}=200)$ respond only slightly even to 0·005 M solutions. It appears that the more rapid the respiration in distilled water, the greater the concentration of succinic acid required to cause a perceptible increase in the respiration rate. A similar position to this obtains with stimulation by potassium chloride.

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* A further possibility is that at low pH the cell membrane is more permeable to ions.

† Xylem tissue, with its lower respiration rate per unit fresh weight (see Table 1) has been inhibited by 0·05 M-succinate.

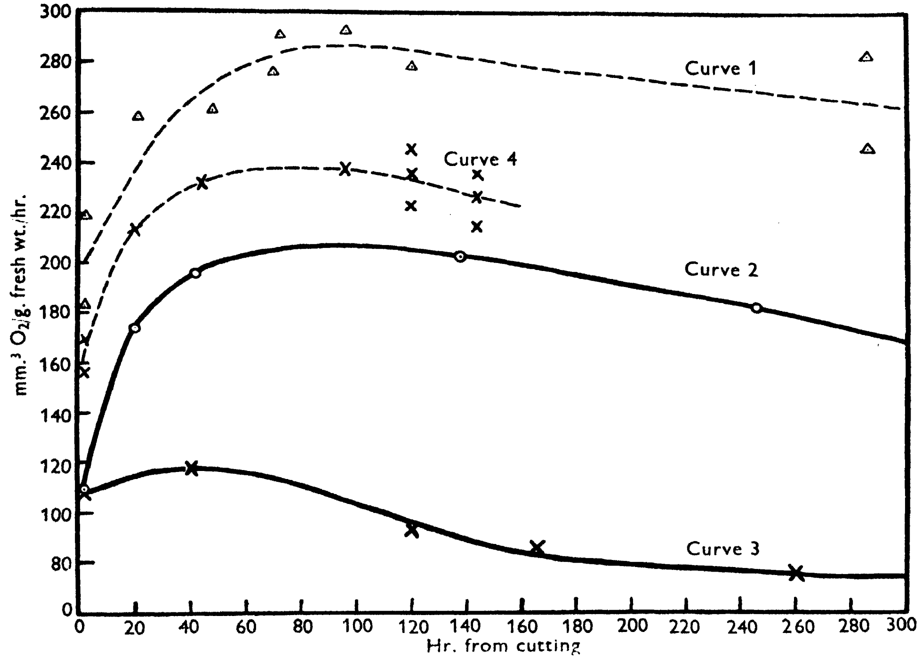

Fig. 6. Carrot tissue; the respiratory drifts with time from cutting of tissue from roots of types A and B. Curve 2, type B, in distilled water; curve 3; type A, in distilled water; curve 1, type B, in 0·05 M-K succinate, pH 4·0–4·5; curve 4, type A, in similar succinate solutions.

A seasonal effect is observed if we compare the effect of 0·05 M-succinate at pH 4 on carrots of different type; the maximum stimulation for carrots of type A is less than that for type B. Moreover, type A carrots, after some months in cold storage, give a smaller response to this concentration than do freshly harvested roots. As indicated in Fig. 6, however, the time-course of maximum succinate respiration is in general similar for all roots. This figure brings out the following facts: During the initial rise in wound respiration, that is to say in the first 80 hr. after cutting, the curve of maximum succinate respiration rises roughly parallel with the curve for respiration in distilled water. Both wound- and succinic-acid respiration are submaximal in any freshly cut carrot tissue, while the succinic-acid respiration in both types of carrot drifts slowly downwards from a peak which is reached at about 100 hr. It should be emphasized that each point in Fig. 6 was obtained from a single Warburg experiment and the disks were not left in succinate solutions for more than a few hours. It is hoped that observations of this type, summarized in Fig. 6, will assist ultimately in the discovery of the true nature of the so-called wound respiration.

In order to determine whether the high respiration rates in succinate solutions were readily reversible, tissue which had been fully stimulated by 0·05 M solution at pH 4, was rinsed with four changes of distilled water, and its respiration in fresh distilled water was followed in the apparatus. The rate of oxygen uptake fell, but not to the initial level and a residual steady stimulation was maintained for the remainder of the experiment. Similar results were obtained when submaximal stimulation had been produced by 0·005 M solutions at pH 4, or by 0·01 M solutions at pH 6. Complete and rapid reversal of oxygen uptake to the initial value was obtained only in tissue which had been very slightly stimulated by 0·001 M solutions at pH 4. Typical results are presented in Fig. 7.

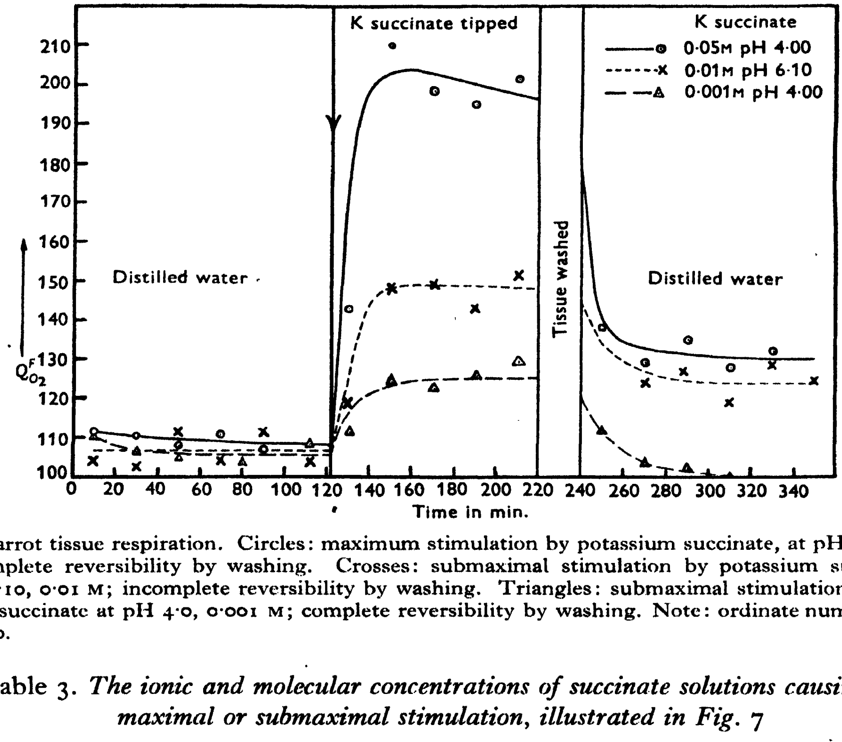

Fig. 7. Carrot tissue respiration. Circles: maximum stimulation by potassium succinate, at pH 4, 0·05 M; incomplete reversibility by washing. Crosses: submaximal stimulation by potassium succinate at pH 6·10, 0·01 M; incomplete reversibility by washing. Triangles: submaximal stimulation by potassium succinate at pH 4·0, 0·001 M; complete reversibility by washing. Note: ordinate numbers begin at 100.

Table 3. *The ionic and molecular concentrations of succinate solutions causing maximal or submaximal stimulation, illustrated in Fig. 7*

Molarity of solution	pH	Total ionic molality	Succinic molecule molarity	Fig. 7
0·05	4·0	0·044	0·03	Circles
0·005	4·0	0·0044	0·003	—
0·01	6·0	0·027	—	Crosses
0·001	4·0	0·0009	0·0006	Triangles

It seems probable that when succinate solutions are added to tissue, at pH 4, the respiratory stimulus has a double origin. Part of it is due to the succinic acid molecule, which enters the cell rapidly by virtue of its lipophilic nature. This effect is quickly abolished by removal of the externally applied succinic acid. On the other hand, salt respiration is known to persist for many hours after the tissue has been washed free of external salts (Robertson & Thorn, 1944). The residual stimulation after washing is therefore presumed to be a salt respiration, induced by the presence of the ions in the succinate solution. In support of this we present in Table 3 the ionic and molecular concentrations present in the experiments illustrated in Fig. 7.

(3) *Succinate and potassium chloride*

Implicit in the hypothesis outlined above is the assumption that respiratory stimulation due to succinic acid molecules is superimposed on a salt respiration associated with accumulation of succinate ions. This 'additive' effect would explain the fact that the optimal stimulation obtained in the presence of potassium succinate solution at pH 4 often greatly exceeds the maximal response to solutions of neutral salts (e.g. KCl). Hence it was desirable to determine whether tissue which was already showing maximal salt effect could be further stimulated by the application of succinic acid.

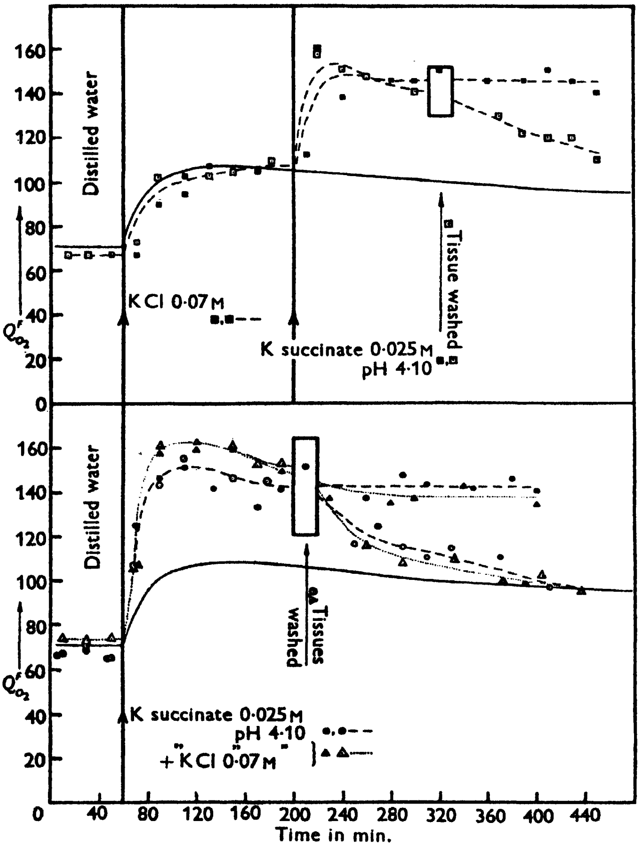

Fig. 8. Carrot tissue respiration; stimulations due to potassium chloride and potassium succinate added together and separately. The solid line, showing potassium chloride-stimulated respiration, were experimentally obtained, but data are omitted for the sake of clarity. Note: this experiment was done with secondary xylem tissue.

Comparisons were made of tissue reactions to the following four solutions: potassium chloride; potassium chloride and potassium succinate (pH 4) added simultaneously; potassium chloride to which potassium succinate was later added; potassium succinate alone. The results are presented in Fig. 8. It will be seen that succinate caused the respiration to increase to approximately the same high level, irrespective of the presence of potassium chloride. When potassium succinate and the mixture of potassium chloride and potassium succinate solutions were removed and the tissues rinsed and returned to distilled water, the respiration rates returned approximately to the level of the salt respiration. These results, therefore, conform with the view presented above, that the optimal succinate stimulation (i.e. that produced by 0·05 M solutions at pH 4) has at least two contributory causes. A salt effect is brought about by the relatively high ionic concentration, and this stimulated rate is further raised owing to the presence of undissociated acid molecules which we presume to be utilized as metabolic substrates or catalysts.

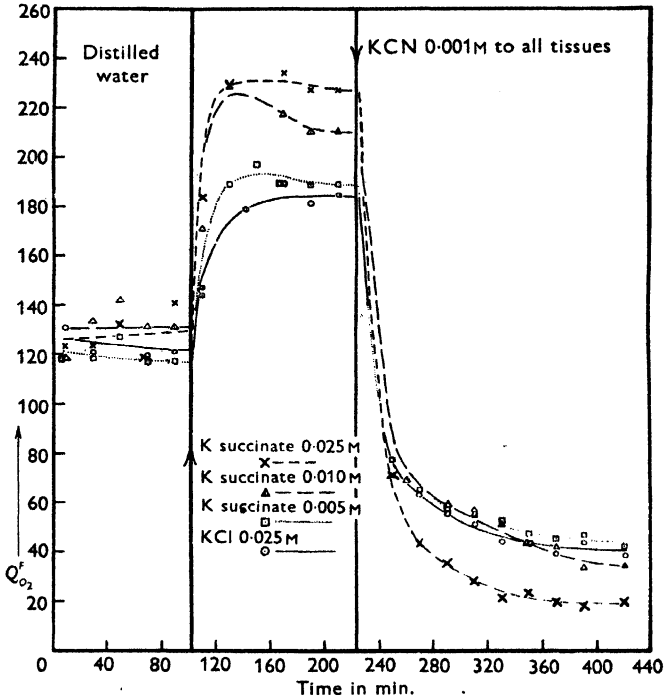

Fig. 9. Carrot tissue; inhibition of potassium chloride- and potassium succinate-stimulated respiration by potassium cyanide, 0·001 M.

(4) *Inhibition*

We have also determined the reaction of stimulated tissue respiration to enzyme inhibitors. It was found that respiration fully stimulated by succinate at pH 4, wound respiration in distilled water, and salt respiration due to potassium chloride, were all inhibited by 0·001 M-KCN.

Salt stimulated and wound-stimulated tissue continued to respire in the cyanide solution (0·001 M) at what we have previously called the ground rate. The respiration of tissue suspended in suboptimal succinate solutions—which yet induced greater stimulation than KCl, as shown in Fig. 9—was also inhibited to the ground level. But in the presence of optimal succinate concentrations, the same cyanide solution (0·001 M) brought the respiration rate below the ground level. In some of these experiments the tissues were killed within 2 hr., the external solution became yellow and the respiration fell to zero.

Comparisons were therefore made of the effect of even lower concentrations of cyanide on tissue fully stimulated by 0·025 M-K succinate at pH 4, and on tissue stimulated by 0·025 M-KCl. It was found that cyanide at 5×10^{-4} M-KCN reduced the respiration of salt-stimulated tissue to the ground level, but again brought the rate in succinate below this. Only in very weak cyanide (1×10^{-4} M-KCN) was the respiration in chloride and succinate inhibited to the same level. This was, however, incomplete inhibition, the respiration remaining above the ground level.

These facts suggest that potassium cyanide inhibits an enzyme system—the succinic acid dehydrogenase-cytochrome system—which is concerned in both salt and succinate stimulations. However, there is the added complexity that when cyanide poisoning prevents the utilization of succinate, high concentrations of this acid (0·02–0·05 M) (possibly because of the lipoid nature of the undissociated molecule), have a deleterious effect on the cell membrane. The membrane structure is apparently disrupted, allowing leakage of cell constituents and a consequent yellowing of the external solution.*

Potassium malonate at pH 4 also depressed the high respiration rates caused by wounding, chloride or succinate, although not always to the basal level. This suggests that succinic dehydrogenase, which presumably is the enzyme immediately concerned in succinic acid metabolism, bears the same close relation to the cytochrome system as it does in animal tissues.

In view of the above facts it seems probable that we shall be unable to use these inhibitors to distinguish between what we shall call the salt component and the metabolic component of succinate stimulated respiration. Nevertheless, the work quoted in this section is of some interest in connexion with our understanding of salt respiration itself. Since maximum succinate respiration is consistently higher than potassium chloride respiration, and since both are inhibited by malonate and cyanide, we may argue that the cytochrome enzyme system cannot be the factor limiting salt respiration induced by potassium chloride. As it is believed that the energy required for salt accumulation is derived from carbohydrate breakdown, the factor limiting salt respiration must be either the endogenous substrate itself or an enzyme which operates prior to the cytochrome system. It would be of interest to determine whether intermediate organic acid metabolites, which are known to stimulate respiration to a higher level than do neutral salts, increase the rate at which these salts may be accumulated. Preliminary experiments of this kind with succinic acid and potassium chloride have been done. The accumulation of the chloride was initially increased when succinate was present, but later the rate of accumulation fell below the control. We used high concentrations of succinic acid (0·05–0·01 M, pH 4) and possibly there is competition for accumulation between chloride and succinate ions. Further work with lower concentrations of succinate ions is desirable, especially since Machlis (1944) has reported that potassium malate, succinate, and citrate, at 0·05 M and pH 5·0, enhance the accumulation of potassium bromide by barley roots.

VI. THE EFFECT OF SUCCINATE ON THE EVOLUTION OF CARBON DIOXIDE

As we shall see in the discussion, succinate may act in several ways as a respiratory stimulant. If this complex position is to be clarified, it is obviously essential to determine the carbon dioxide output of treated tissues simultaneously with the oxygen uptake. The importance of R.Q. determination has been stressed by Bennet-Clark & Bexon (1943), but has often been overlooked by other workers dealing with plant reactions to organic acids.

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* We have already pointed out (p. 156) that high succinate concentrations alone (about 0·1 M) have a similar effect.

(1) *Succinate addition at* pH 4

In all our experiments in which succinate was applied to the tissue at pH 4, increased respiratory quotients were observed. Fig. 10 illustrates a typical experiment in which we recorded the simultaneous absorption of oxygen and the evolution of carbon dioxide. In this experiment the mean R.Q. in 0·05 M-succinate at pH 4 was 2·13.

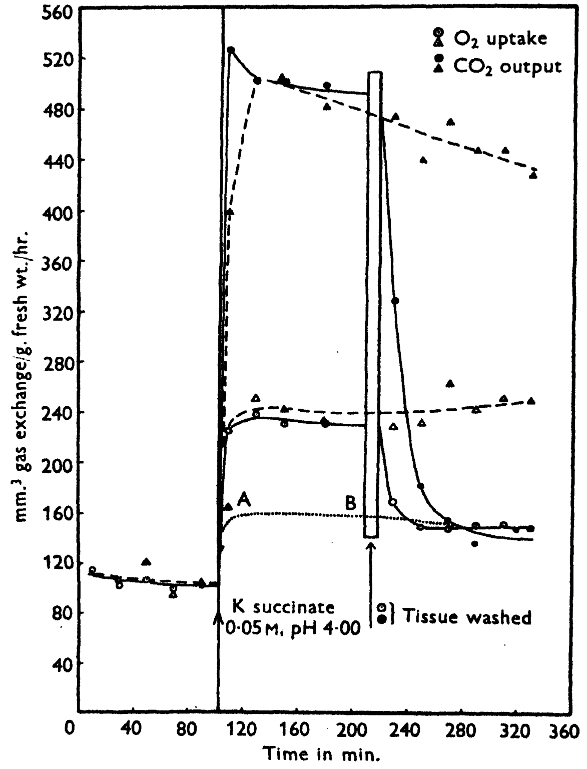

Fig. 10. Carrot tissue; effect of potassium succinate, pH 4·0, 0·05 M, on rate of gas exchange. (The R.Q. in distilled water for the first 100 min. was 0·97.) Circles: these two sets of tissue slices were washed and replaced in distilled water. Triangles: these tissue slices remained in potassium succinate. The dotted line *AB* represents the average level of salt-stimulated respiration.

It will be realized that a sudden decrease in pH from 5·5 (distilled water surrounding living disks) to 4·0, by the addition of any acid, is liable to cause a rapid evolution of carbon dioxide, owing in the main to the liberation of bound CO_2 and also to the decreased solubility of this gas in acid solutions. Preliminary experiments showed the magnitude of this effect. These were carried out by tipping on to tissue slices a potassium chloride-hydrogen chloride solution adjusted to pH 4 and having a final chloride ion concentration of 0·05 M. This solution caused an increased salt respiration, the R.Q. of which was assumed to be unaltered from the normal (1·0).* There was, however, a sudden gush, complete within 15 min., of about 30 cu.mm. CO_2/g. fresh weight of carrot tissue. Slices subjected to different conditions probably contain different amounts of carbon dioxide in solution and bound as bicarbonate. However, when normally respiring tissue was killed by acidification with 0·1 N-HCl, between 30 and 40 cu.mm. CO_2/g. fresh weight were evolved. (Turner in 1938 reported a similar figure.) We may, therefore, consider that for the first 15 min. after the tipping of an acid solution, some 30 cu.mm. of carbon dioxide are likely to be expelled by non-respiratory reactions. (These may occur only on the cell surfaces for it is unlikely that the internal cell pH is rapidly affected by external changes.) This volume would appreciably increase the average R.Q., so in all our calculations of mean R.Q. in solutions of pH 4, we have neglected the first 20 min. readings.

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* A characteristic feature of salt respiration due to potassium chloride alone is the unchanged R.Q.

(*a*) *Concentration and* R.Q.

Because of the high R.Q. figure obtained in preliminary experiments, we carried out a series of measurements of the R.Q. in succinate solutions at pH 4, but at varied concentration. In Fig. 11 we plot the drift in the R.Q. measured at 20 or 40 min. intervals over experimental periods of 2–4 hr. At all concentrations used, the initial gush of CO_2 referred to above was observed. The graph shows that the tissue R.Q. approached unity only in the presence of succinate solutions too dilute to induce maximum stimulation of oxygen uptake.

It also becomes apparent from Figs. 11 and 13 that in 0·05 M succinate solutions (which cause maximum oxygen uptake) the high R.Q. was maintained for as long as 4¼ hr.; the value never fell below 1·8. A high steady rate of gas exchange was similarly recorded in the presence of 0·01 M solutions.* However, Figs. 11 and 12 show that in the presence of solutions causing submaximum stimulation of oxygen uptake, the R.Q. gradually fell to unity in spite of the fact that the rate of oxygen uptake remained steady.

(*b*) *Reversibility*

When tissue, which was respiring in the presence of 0·05 M–0·01 M solutions at pH 4, was thoroughly washed and replaced in distilled water, the R.Q. always fell rapidly, returning within about 1½ hr. to its normal value of 1 ± 0·05. This behaviour is illustrated in Fig. 10. During this fall in R.Q., the oxygen uptake returned to a steady value above the ground rate and characteristic, for this tissue, of salt respiration. We, therefore, assume that during maximal stimulation by succinate at pH 4 there occurs, simultaneously: (i) normal respiration, R.Q. 1·0; (ii) stimulated salt respiration, R.Q. 1·0; and (iii) a succinate-stimulated gas exchange with a net R.Q. of about 4·0. The gross R.Q. for total gas exchange in (*a*), (*b*) and (*c*) is approximately 2·0. Thus in Fig. 10 the dotted line *AB* represents the presumed salt-plus-ground-respiration going on in the presence of succinate. The ratio of excess CO_2 output to oxygen uptake above this level is 4 in this experiment. In similar experiments a mean value of 4 has been observed.

(*c*) *The effect of acids related to succinic*

It was thought that it might be possible to demonstrate the salt-respiration fraction of succinate stimulation as follows. If the tissues were provided with a solution of an acid closely related to succinic, having approximately the same degree of dissociation, then conceivably the ions would induce salt respiration, the molecules being inactive, owing to the specificity of succinic dehydrogenase. If so, some stimulation of gas exchange should occur, the R.Q. remaining unaltered. In order to test this possibility, we compared the

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* The results recorded in Fig. 13 were from many series of experiments carried out at intervals over 2 years.

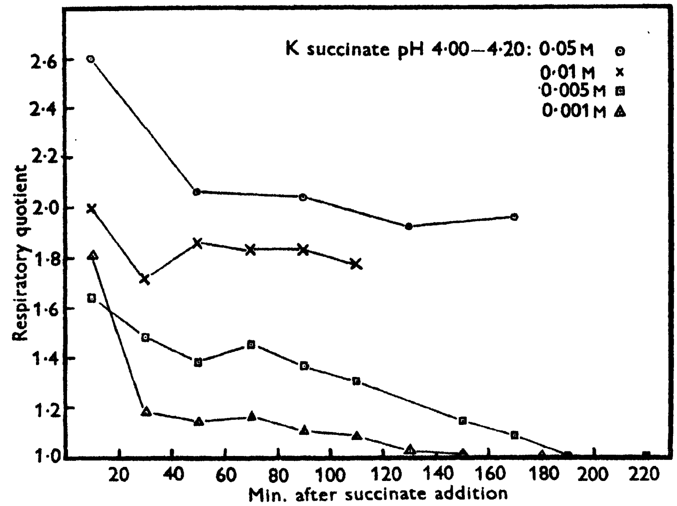

Fig. 11. Carrot tissue; drift in R.Q. values from the instant of tipping succinate solutions.

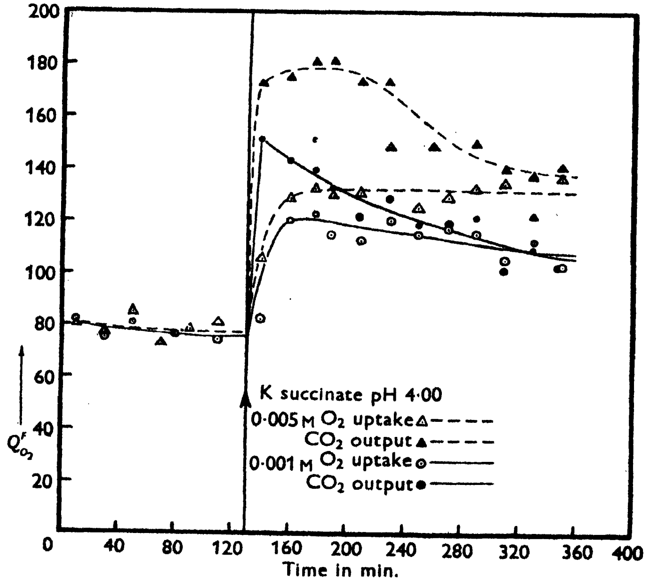

Fig. 12. Carrot tissue; the rates of O_2 uptake and CO_2 output of submaximally stimulated tissue, showing the return of the R.Q. to unity after 4 hr.

effects of three acids: adipic, glutaric and mono-methyl-succinic. Solutions of these acids were adjusted to 0·025 M and pH 4·30, and were added to normally respiring carrot tissue. Contrary to expectation, each acid killed the tissue rapidly, the external solutions becoming yellow.* More dilute solutions of mono-methyl-succinic acid were proportionately less toxic, 0·001 M producing only slight, gradual inhibition. It is concluded that adipic, glutaric and mono-methyl-succinic acid are not metabolized by succinic dehydrogenase and cause disruption of the cell membrane, so that their ions are unable to participate in salt respiration. (It may be noted here that little work has been reported on the effect of substituted succinic acids on respiration, but Nord & Vitucci (1947) state that wood-destroying fungi are able to metabolize di-methyl succinic acid which is unlikely to act as a substrate for succinic dehydrogenase *in vitro* (cf. Gottschalk, 1941).

(2) *Succinate addition at* pH *greater than* 4·0

It has already been shown that as the pH of the succinate solution increases there is a progressively smaller stimulation of the oxygen uptake. The respiratory quotients in the pH range 5–7 were also measured and the results are presented in Fig. 13; in the accompanying Table 4 are set out the molecular and ionic concentrations present in the different solutions.

Table 4. *Ionic and molecular concentrations present in succinic acid solutions adjusted with potassium hydroxide to different* pH *values*

pH	[Molecule] (Molar)	[H. succinate^{-}] (Molal)	[Succinate$^{=}$] (Molal)	[K^{+}] (Molal)
		(1) 0·05 M solutions		
4–4·2	0·03–0·025	0·02–0·025	0·001–0·002	0·023
5·0	0·005	0·036	0·009	0·054
5·4	0·002	0·029	0·02	0·068
6·85	—	0·003	0·047	0·095
7·0	—	0·002	0·048	0·098
		(2) 0·01 M solutions		
4·0	0·006	0·004	0·0002	0·005
5·0	0·001	0·007	0·002	0·011
5·4	0·0004	0·006	0·004	0·014
6·1	—	0·0022	0·0075	0·0172

Fig. 13 shows the results obtained with two strengths of succinate solutions, 0·05 M and 0·01 M. The R.Q. consistently fell as the pH was raised, reaching the low figure of 0·70–0·80 in either solution at pH 6–7. These figures were obtained by the direct method and they are probably low owing to some CO_2 retention at these pH values. In the solutions of pH 6, however, the R.Q. remained steady at about 0·8 over 280 min., during which the pH fell to a value near 5·5. This fact and check experiments by the acid-tip method show that the R.Q. is significantly below 1·0 when succinate is applied at a pH from 6 to 7.

The low-respiratory quotients in neutral potassium succinate solutions support the idea that in the less acid media the tissue preferentially absorbs potassium ions with the accompanying Ulrich effect of increased organic acid formation. Neutral solutions of potassium succinate contain concentrations of cations and anions in the ratio approximately 2 : 1, since, at this pH, divalent succinate ions predominate. Hence it might be expected that the highly mobile K^+, which has been shown by the use of radio-active isotopes to penetrate cell membranes rapidly, would be absorbed in excess of organic anions even if these were actively accumulated by a metabolic process.

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* The inhibition due to these three acids is different in kind from that caused by malonic acid, which does not inhibit ground respiration or bring about yellowing of the solution. This acid is believed to act by combining with the active centres of succinic dehydrogenase.

Machlis (1944), too, reported an Ulrich effect when he tested the responses of excised barley roots towards organic acids. With tissue which had been in potassium-succinate solutions for more than 3 hr., he obtained a steady R.Q. of 0·90; the oxygen absorption had meanwhile risen 34% above the control rate. This finding is somewhat surprising because Machlis used 0·05 M solution at pH 5; this should contain sufficient undissociated succinic acid to allow succinate metabolism with a high R.Q. It is, however, possible that the metabolism of this acid by barley-root tissue is different from that observed in the storage roots of beet and carrot.

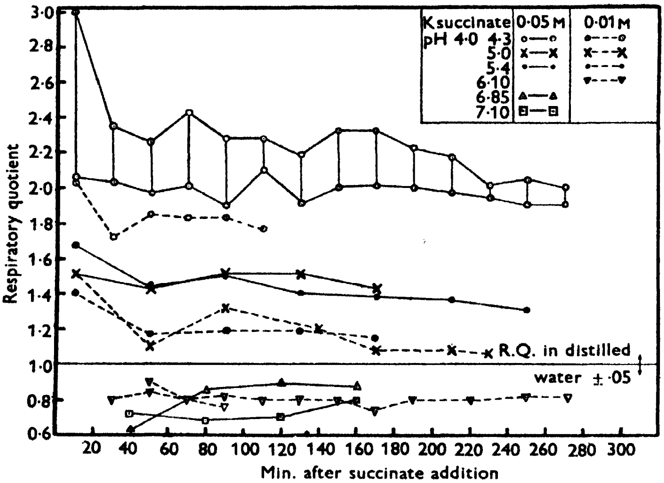

Fig. 13. Carrot tissue; drift in R.Q. values in succinate solutions of different pH values; comparison of 0·05 M, and 0·01 M solutions.

VII. PRELIMINARY EXPERIMENTS ON THE UPTAKE OF SUCCINIC ACID

Experiments have been carried out to discover whether the uptake of succinic acid by carrot tissue is a function of pH. Carrot slices were placed in succinic-acid solutions adjusted to two different pH values and the loss of acid from the two solutions was measured. The R.Q. of duplicate sets of tissue was also measured over the same time interval (4 hr. 20 min.). One set of tissue slices was in 0·0215 M-K succinate solution at pH 4·4, the other set in 0·0215 M-K succinate at pH 6·9. The solutions bathing the tissues were finally decanted and the total acid in each was determined electrometrically.

The following method was adopted: The succinic acid solution was accurately diluted 125 times with dilute hydrochloric acid which lowered the pH to 4·0 thereby depressing the ionization of succinic acid. This solution was then titrated against standard alkali, the pH being measured with a glass electrode after each additional drop. From these measurements a sigmoid acid dissociation curve was drawn, characteristic of succinic acid. The titration end-point was always taken at pH 8·57, for at this pH, the amount of succinate left untitrated was only one in one thousand. Moreover, the alkali error (the volume of alkali required to increase the pH from 7 to 8·6) is negligible. This method actually measures the dissociable hydrogen ions, which for succinic acid would correspond with twice the number of succinate radicles present. Although the experimentally determined dissociation curve is characteristic of, it is not specific for, succinic acid. Other dibasic acids with similar pK_2 values could not be distinguished if they were also in solution (e.g. by leaching or exchange). Oxalic and malonic acids, however, if present in appreciable concentrations, would modify the shape of the curve. Glutaric and higher dicarboxylic acids would be masked, but their presence is improbable in view of their pronounced toxicity.

It was also felt desirable to determine the concentration of acid in solutions which had been in contact with tissue for only a short period—it was possible that some dilution might occur from effluence of water present in intercellular spaces of the disks. Ten minutes was chosen as being too short for metabolic changes to have become appreciable, but long enough for the initial equilibrium between tissue and solution to have been approached.

The results of the experiment are presented in Table 5.

Table 5. *Changes in* pH *and in the concentration of dissociable* H^+ *after contact of succinate solutions with respiring carrot tissue*

(Initial concentration of succinate ([dissociable H^+]) 0·0215 M)

(1) Initial pH*	(2) Final pH after 4 hr. 20 min.	(3) R.Q., mean during 4 hr. 20 min.	(4) [Dissociable H^+] after 10 min. = *A*	(5) [Dissociable H^+] after 4 hr. 20 min. = *B*	(6) *A*–*B* = acid disappearance
4·4	4·9	2·0	0·0220	0·0130	0·0093 M
6·9	6·3	0·8	0·0265	0·0217	0·0048 M

* This refers to the pH of the solution initially in contact with tissue: before titration the pH was adjusted to 4·0 with HCl.

It will be seen from Table 5 column (2), that the pH of the more acid solution rose while that of the less acid solution fell. Parallel experiments showed that in each solution the final pH was achieved in less than 2 hr. Column (4) shows that in each solution the acidity (i.e. dissociable H^+ concentration) had risen by the end of 10 min., but the increase was only significant for the less acid solution. Since it is to be expected that the intercellular water would dilute the acid, there must be some process occurring which concentrates the acid. There could be two such processes: (1) At pH 6·9 the K^+ concentration is 0·042 M, greatly in excess of the other ionic species present. Free exchange of the K^+ with the H^+ of the tissues could take place. (2) Rapid intake of divalent succinate ion (0·021 M) could have occurred. However, the first process is the more likely as it is known that divalent ions penetrate cell membranes slowly, while the concept of K^+–H^+ exchange is supported by the low R.Q. (column (3)).

Column (6) records the actual disappearance of dissociable H^+ from the solution over the whole experimental period. Since we believe that H^+ is lost from the tissues during the first 10 min. it seems reasonable to suppose that it must have been transported back again in combined form: i.e. either as H_2S or as the monobasic ion HS′. The disappearance of both the molecule and the ion (HS′) of succinic acid from solution at pH 4·4 would explain the rise in the pH.

For the solution at pH 6·9 the initially increased [H^+] would favour the formation of HS', and we assume that the acid disappearance recorded in column (6) must have been due to the intake of this ion. It was about half the loss from the solution at pH 4.

These preliminary results, although indirect, therefore support the hypothesis put forward in earlier sections of this paper. Confirmation must await more direct analyses.

VIII. DISCUSSION

The results reported indicate that the respiratory response of carrot root tissue to potassium succinate is most complex. It is evident that a full knowledge of the reactions involved will only be obtained when measurements of gas exchange under various conditions are supplemented by rigid chemical analyses probably involving the use of isotopes. However, enough data are already available to make certain conclusions possible.

The succinate effect varies with the pH in such a way that, within the physiological range, the stimulation of oxygen uptake is attributable to two separate causes: (*a*) ionic, (*b*) molecular. Each of these effects is itself complex and must be considered separately.

The ionic effect at pH 4 consists of an enhancement of respiration, associated probably with an accumulation of ions into the cell. We have as yet no direct evidence for such accumulation, but part of the respiratory behaviour is analogous to that shown by solutions of potassium chloride. For example, this part of the respiration is not rapidly reversed by washing, and it is sensitive to potassium cyanide, its R.Q. is 1·0. If we follow Lundegårdh's (1946) hypothesis for the mechanism of the salt effect we must assume that this ionic accumulation (i.e. of one or both succinate ions) effects a more rapid turnover of the cytochrome system, Fe^{++} cytochrome-C itself acting as a carrier of the anions. However, since cytochrome-C is reduced by the succinic dehydrogenase-succinic acid system we shall assume provisionally that those succinate ions which are transported by the cytochrome cannot at the same time take part in the reduction of the cytochrome by the activity of succinic dehydrogenase (see Fig. 14).

The ionic effect at pH greater than 4 is assumed to be largely a salt effect, but it is further complicated by the excess absorption of K^+ leading to the Ulrich effect which has already been discussed and which is accompanied by a low R.Q. (Fig. 14A).

By molecular effect we mean the influence on the gas exchange due initially to the entry of succinic acid molecule itself into the cell. This effect is naturally greatest at pH 4 when the molecule is present in highest concentration in succinate solutions. We presume that on entering the cell the acid is dissociated to a degree determined by the pH of the cytoplasm. Its ions can then be utilized directly as substrates for succinic dehydrogenase (cf. Potter & Dubois, 1943), (Fig. 14C). This effect is rapidly abolished by washing, it is sensitive to potassium cyanide and to malonate; and is probably not catalytic. However, the final fate of the succinate ions remains obscure. We presume them to take part in some organic acid cycle, such as the Krebs cycle, and to be converted at least as far as malic acid. The peculiar feature emerges, however, that the R.Q. of succinic acid utilization (i.e. excluding basal and salt respiration) is in the region of 4. It is pertinent at this point to discuss the important work by Bennet-Clark & Bexon. They postulate that in untreated tissue a large excess of malate is stored in the vacuole, from which it slowly diffuses into the cytoplasm. Here an anabolic process is presumed to occur, as in equation (*a*). The sugar formed passes back into the vacuole where an opposed catabolic process occurs, the overall R.Q. being maintained at 1·0.

(*a*) Malic acid → sugar + CO_2 (cytoplasm),

(*b*) Sugar + O_2 → malic acid (vacuole).

When malate is supplied externally to the cell it is supposed to be anabolized by process (*a*) at a greater rate than the cycle can cope with, and consequently a high R.Q. is maintained.

This hypothesis adequately explains the facts as regards malate stimulation, but Bennet-Clark & Bexon go further and suggest an identical explanation of the stimulation and high R.Q. given by both succinic and citric acids. In their table which relates 'extra CO_2' to acid disappearance they give similar ratios for all three acids. Yet if citric and succinic are first converted to malic (e.g. via the Krebs cycle) we have a gaseous exchange represented as in (*c*) and (*d*).

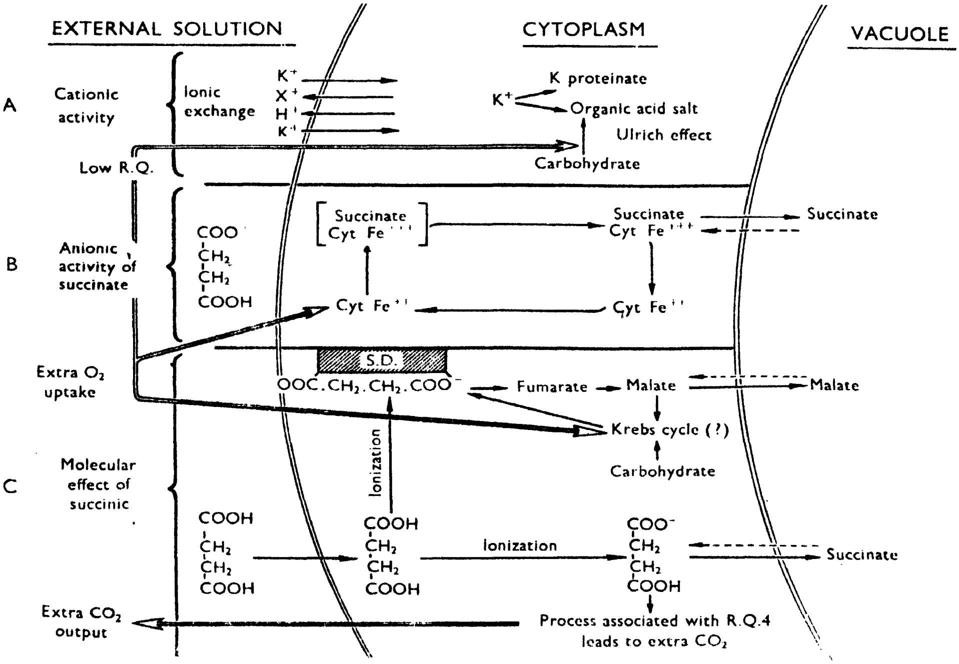

Fig. 14. Diagrammatic representation of probable cellular activities affecting the metabolism of potassium succinate over the experimental range in pH, 4–8. Areas A and B: reactions induced by ions, constituting 'salt respiration'; and change in organic acid balance within the cell ('Ulrich effect'). In area B 'cyt Fe^{+++}' indicates cytochrome C in the oxidized form, and 'cyt Fe^{++}', in the reduced form. This cycle has been suggested as a mechanism of salt accumulation (Lundegårdh, 1946); see Robertson & Wilkins, 1948). Area C: reaction induced by penetration of undissociated succinic acid through the ectoplast. After entering, the molecules dissociate to a degree dictated by the pH of the cell cytoplasm. 'S.D.' represents succinic dehydrogenase. Vacuole area: probably contains malic and succinic acids, as well as citric and fumaric (the latter have been omitted from the diagram for the sake of clarity). These four acids have been shown by paper chromatography to occur in carrot tissue extracts (Lugg & Overell, 1948) and may therefore occur in solution in the vacuole.

(*c*) Citric ($C_6H_8O_7$) + 3O → malic ($C_4H_6O_5$) + $2CO_2$ + H_2O,

(*d*) Succinic ($C_4H_6O_4$) + O → malic ($C_4H_6O_5$).

Thus the extra CO_2 evolved (i.e. in excess of O_2 uptake) would not be the same for each acid.

Bennet-Clark & Bexon refer throughout to 'gross R.Q.' which is the ratio of the total CO_2 output to total O_2 uptake in acid stimulated slices, and they have taken no account of salt respiration. Even so, as they point out, the ratio is higher than it would be if the acids were completely oxidized. We prefer to measure the R.Q. of the true 'acid' metabolism, as the gross R.Q. is only the mean of three ratios. In all our experiments the true R.Q. of the 'acid' metabolism is about 4.

One possible explanation of a high R.Q. (neglected by Bennet-Clark) is that the excess acid is converted to pyruvic and then decarboxylated, the acetaldehyde so formed being further reduced by coenzyme I to alcohol. The overall equation for this reaction may be written as

$$2C_4H_6O_4 + O_2 \rightarrow 2C_2H_6O + 4CO_2; \text{ R.Q.} = 4.$$

In order to test this possibility, carrot disks were aerated in potassium succinate at pH 4 for some hours, and the solution then tested for alcohol by the micromethod of Widmark (cf. Gottschalk, 1941). However, no alcohol could be detected. This was not unexpected, since work with baker's yeast respiring glucose has shown that the reduction of acetaldehyde to alcohol in the presence of oxygen is negligible, owing to the prevailing activity of the oxidizing enzyme systems. The reason for the maintenance of an R.Q. of 4, therefore, is still obscure. However, further work is being done to determine the end product of succinate metabolism. One possibility is that the extra CO_2 does not arise from succinic acid itself, but is due rather to the excess of this substance upsetting normal metabolism and allowing the production of CO_2 from the decarboxylation of some as yet unknown substrate. As the R.Q. shows reasonable constancy at 4 for the same experimental conditions, we postulate some stoichiometric relationship between the decarboxylation reaction and the added succinate. The R.Q. in our experiments is too high to be in accord with the particular concept of oxidative anabolism set out in equation (*e*).

$$(e)\ 2\ \text{succinic} + O_2 \rightarrow 2\ \text{malic} \rightarrow 2\ \text{sugar} + 2CO_2.$$

IX. SUMMARY

1. Succinic acid, adjusted to various pH values with potassium hydroxide, has been applied to thin tissue slices of the carrot root and the subsequent respiratory behaviour has been followed.
2. The type of response to this acid varies with the concentration and the pH.
3. At low pH the acid causes maximal stimulation of the oxygen uptake and this is accompanied by a gross R.Q. of 2·0.
4. At high pH the stimulation of oxygen uptake is less; the R.Q. is usually less than unity.
5. The rate of oxygen uptake and the R.Q. show marked changes when tissue stimulated by succinate is rinsed and replaced in distilled water.
6. It is concluded that the metabolic responses are complex; and must be interpreted in terms of salt respiration, cation exchange; and 'molecular activity' as summarized in Fig. 14.
7. Analysis of the results in these terms suggests that excess succinic acid at low pH stimulates a process having a net R.Q. of 4, which remains to be elucidated.
8. It is clear that succinic acid is a metabolite in the plant tissue studied, but our results provide no direct evidence for the existence of the complete Krebs cycle in this tissue.

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REFERENCES

Anderson, E. H. (1945). Studies on the metabolism of *Prototheca Zopfii*. *J. Gen. Physiol.* **28**, 297.

Bennet-Clark, T. A. & Bexon, D. (1943). Water relations of plant cells. III. Respiration of plasmolysed tissue. *New Phytol.* **42**, 65.

Conway, E. J. & Brady, T. (1947). Mechanism of high acid production by yeast. *Nature, Lond.*, **159**, 137.

Dixon, K. C. (1943). *Manometric Methods*, 2nd ed. Cambridge University Press.

Gottschalk, A. (1941). Mechanism of the Pasteur effect in alcoholic fermentation by yeast. *Aust. J. Exp. Biol. Med. Sci.* **19**, 211.

Higginbotham, L. & Lapworth, A. (1922). The formation of substituted succinic acids from esters of unsaturated acids. *J. Chem. Soc.* **121**, 49.

Krebs, H. A. (1935). Metabolism of amino-acids. III. *Biochem. J.* **29**, 1633.

Lugg, J. W. H. & Overell, B. T. (1948). Partition chromatographic separation of organic acids on an inert support. *Aust. J. Sci. Res.*, Part A, **1**, 98.

Lundegårdh, H. (1946). Transport of water and salts in plant tissues. *Nature, Lond.*, **197**, 575.

Machlis, L. (1944). The influence of some respiratory inhibitors on respiration and salt accumulation in excised barley roots. *Amer. J. Bot.* **31**, 183.

Myers, J. (1947). Oxidative assimilation in relation to photosynthesis in *Chlorella*. *J. Gen. Physiol.* **30**, 217.

Nord, F. F. & Vitucci, J. C. (1947). Enzymic studies of the mechanism of wood decay. *Nature, Lond.*, **160**, 224.

Osterhout, W. J. V. (1925). Is living protoplasm permeable to ions? *J. Gen. Physiol.* **8**, 131.

Petrie, A. H. K. (1938). Intake of ions by carrot tissue at different pH. *New Phytol.* **37**, 211.

Potter, V. R. & Dubois, K. D. (1943). VI. Inhibitor studies with succinic dehydrogenase. *J. Gen. Physiol.* **24**, 391.

Robertson, R. N. R. & Thorn, M. (1944). Studies in the metabolism of plant cells. 4. Reversibility of the salt respiration. *Aust. J. Exp. Biol. Med. Sci.* **23**, 305.

Robertson, R. N. R. & Wilkins, M. (1948). Studies in the metabolism of plant cells. 7. Quantitative relation between salt accumulation and salt respiration. *Aust. J. Sci. Res.*, Part B, **1**, 17.

Thomas, L. A. (1930). Respiration of carrot tissue in buffer solutions. *Aust. J. Exp. Biol. Med. Sci.* **8**, 225.

Turner, J. S. (1938). The effect of mono-iodoacetate on respiration and fermentation. *New Phytol.* **37**, 232.

Turner, J. S. (1940). Respiratory metabolism of carrot tissue. III. *Aust. J. Exp. Biol. Med. Sci.* **18**, 273.

Turner, J. S. & Hanly, V. F. (1947). Malonate and plant respiration. *Nature, Lond.*, **160**, 296.

Ulrich, A. (1941). Metabolism of organic acids in excised barley roots as related to cation-anion balance during salt accumulation. *Amer. J. Bot.* **28**, 526.

Warburg, O. & Negelein, E. (1920). Über die Reduktion der Saltpetersäure in grünen Zellen. *Biochem. Z.* **110**, 66.

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THE AMINO-ACID PRECURSORS OF THE BELLADONNA ALKALOIDS

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(With 1 figure in the text)

It is generally supposed that the nitrogen of the alkaloids is derived from the nitrogen of amino-acids in the plants that form them. A first step in elucidating the stages in their biosynthesis must therefore be an identification of the amino-acid or acids from which they derive their nitrogen. Of the amino-acids known to occur more or less universally in plant proteins, two have been proposed on general chemical grounds as possible precursors of the belladonna alkaloids, chiefly *l*-hyoscyamine. They are proline (Weevers, 1933) and arginine (Robinson, 1917). Other amino-acids might be added, and any one of them might become remote precursors via the release of ammonia by oxidation. In a series of experiments lasting over five seasons, ten different amino-acids have been examined.

METHODS

Synthesis of solanaceous alkaloids appears to occur principally in young, actively growing tissues (cf. James, 1946). In many species it is most vigorous in young roots. The proper handling of root systems to obtain numerous comparable samples is very difficult and has not yet been satisfactorily achieved. We have therefore preferred to use detached leaves, having found with belladonna that increases of alkaloid could be shown to occur in them.

Sampling. Since the methods of estimating alkaloids entail the destruction of the tissue containing them, we were obliged to compare different samples of tissue before and after treatment. Samples to be compared had to consist of leaves all at a similar stage of development, because the ability to synthesize alkaloids appears to depend markedly upon age. Fortunately, the metabolic age of a leaf could be decided within limits by its appearance and position on the axis. There is probably a loss of metabolic activity at any given position on the axis as the season advances (cf. Richards, 1934) and the differences between leaf samples collected on successive occasions are likely to be greater than the differences between samples taken on a single occasion. In practice, our leaf samples have consisted of a given number of leaves taken from a predetermined position on the stem, so graded that each sample had an equal fresh weight as picked. Variations of fresh weight were kept below 5% on any occasion; but might be much greater between different occasions. Direct numerical comparisons are made only between samples taken on a single occasion.

Feeding. The only method that promised much prospect of success in identifying alkaloid precursors was a suitable feeding technique. A number of possible variations were tried and the following adopted for nearly all the experiments to be described.

A large number of belladonna leaves were collected from cultivated plants of equal age. The leaves were taken only from a predetermined zone on the stem, which varied according to the experiment in hand. Usually, in order to get a large enough sample from similar plants, the picking zone had to include two or three nodes. The leaves were put into a tin as picked and conveyed as rapidly as possible to the laboratory. There they were graded into the number of samples required, all of equal fresh weight to the nearest gram. The number of leaves in a sample varied from fifteen in experiments with fully developed leaves to fifty in experiments with very small, young leaves. Each set of leaves was arranged in a bunch with the petioles loosely held together by a rubber band. The petioles were trimmed to uniform length with a sharp razor and the fresh surface was immediately immersed in the feeding solution. By careful arrangement of the leaves, the amount of petiole trimmed off could be kept down to negligible proportions.

The feeding solutions were prepared beforehand and the desired amount, usually 20 ml., was pipetted into a 100 ml. beaker. Each sample of leaves was placed in a separate beaker and the whole batch put into an incubator at 25° C. Solutions were removed and fresh ones put in their place after 24 or 48 hr. The total duration of the experiment was usually 3 or 4 days, since initial experiments showed that leaves on distilled water or sucrose solution remained healthy and did not appreciably change their alkaloid content during 3 days at 25° C. Uptake of solutions into the leaves was usually rapid and could be estimated approximately by measuring the solution remaining and, hence, the amount removed. This afforded only a rough estimate, since some direct evaporation from the liquid surface is likely to have occurred.

One sample of leaves was always dried immediately in a forced current of air at 50° C. The samples were thinly spread and dried to a crisp and bright green condition in a few hours. At the end of the 3 days the other samples were similarly dried and the dry weights at 50° C. and the alkaloid contents determined. Samples were kept in air-tight tins while waiting their turn for analysis and were re-dried at 50° C. immediately before use.

Assay. The alkaloid content (assay) is expressed as gram *l*-hyoscyamine in 100 g. of the dried material. It was determined in the earlier experiments by the colorimetric method of Allport & Wilson (1939) which depends on the Vitali-Morin reaction. The specificity of this reaction was examined and described in an earlier paper (James & Roberts, 1945). The production of purple colours under the conditions used was shown to be restricted to the esters of tropic acid and intense colours could only be obtained with its three alkaloid esters, *l*-hyoscyamine, *l*-hyoscine and *dl*-atropine, i.e. the alkaloids of belladonna leaf. Assays of total alkaloids in belladonna leaf by the Allport & Wilson technique were found to agree well with the results obtained by the official method of the British Pharmacopoeia (Watson, Heard & James, 1941).

In later experiments use was made of the method of Roberts & James (1947). After initial purification on adsorption columns, the alkaloids are estimated in this method by microtitration. A small aliquot of the purified extract is also submitted to Vitali-Morin treatment to determine its alkaloids colorimetrically. In normal samples of belladonna leaf the colorimetric estimation (tropic esters) and the titration (adsorbable bases) agree closely. Comparison of the two estimates has often proved its value. As might be expected, the colorimetric estimation never greatly exceeds the titration, but non-alkaloidal bases are sometimes revealed by a high titration value in excess of the colour estimation. Comparing the colour values obtained from belladonna leaves by the Allport & Wilson method with those of the Roberts & James technique, it is frequently found that the former is slightly (about 5%) higher. This might be due to traces of other colours or slight cloudiness concealed in the Vitali purple obtained with the less purified extract.

Where good agreement is obtained between the colorimetric and titrimetric results, the estimate of alkaloid content may be accepted with considerable confidence. On a number of occasions, *l*-hyoscyamine and *l*-hyoscine have been isolated from our leaves and identified as picrates (cf. p. 180). As it has always been necessary to work with material containing large initial amounts of alkaloid, it is evident that such qualitative isolations can only be of secondary importance and that the principal reliance must be placed upon trustworthy estimation.

Quantity of alkaloid in the leaves. The analytical results are necessarily expressed as a percentage of the total dry matter. Variations in this ratio may arise from changes in any of the leaf constituents. It is well known that during periods of isolation the soluble carbohydrates of the leaf are consumed to such an extent that its dry weight falls appreciably. The alkaloid percentage is correspondingly increased and such increases do not necessarily imply any actual synthesis. To establish the occurrence of synthesis it is necessary to show that the actual amount of alkaloid in the leaf has increased, and this can only be done by comparing the total amounts in equal samples. As our samples were arranged to have both equal numbers of leaves and equal initial fresh weights, it is immaterial whether the amounts of alkaloids are calculated per leaf or by initial weight. In the following tables the results are usually given as mg. *l*-hyoscyamine per leaf: occasionally also as mg. *l*-hyoscyamine in 100 g. initial fresh weight. This is conveniently computed as $1000\,Aw/W$, where A is the analytical result (assay), w the dry weight at the time of assay and W the initial fresh weight of the sample. For the statistical analyses this latter basis is somewhat the more convenient and has been used throughout. Although it is necessary to calculate the weight of the alkaloids according to the molecular weight of the predominant *l*-hyoscyamine, the methods include the two closely allied alkaloids which probably contribute 15–20% of the whole (cf. Rowson, 1944).

Nitrogen determinations. In some experiments, determinations were also made of the total soluble and protein nitrogen. Disks were cut between the veins of the leaves with a well-sharpened 5 mm. cork-borer, one from each side of the midrib. Each leaf was sampled in this way and the residues used for the alkaloid determinations. The disks were mixed and divided into two equal aliquots, each of which was weighed. The first aliquot was dried at 100° C., and after weighing was used for the determination of total nitrogen. The second aliquot was covered with 24 ml. boiling distilled water in a boiling tube and heated at 100° C. for 1 hr., with frequent stirring. It was then cooled and made up to volume with distilled water, after which 8 ml. 40% trichloracetic acid were added, stirred well, and the mixture allowed to stand for 20 min. The supernatant liquid was decanted and a second mixture of 24 ml. 40% acetic acid added to the residue. After a further 20 min., this was decanted into the first portion. The combined decantates were evaporated slowly under reduced pressure to 10 ml. and used for the determination of soluble nitrogen. Protein nitrogen was determined in the residue.

The nitrogen in each of the categories, total, soluble and protein, was determined by micro-Kjeldahl after digestion with the phosphoric-sulphuric-persulphate mixture of van Slyke (Peters & van Slyke, 1932). Good agreement was usually obtained between the estimates for total nitrogen and the sum of protein nitrogen + soluble nitrogen.

RESULTS

Preliminary experiments. In the initial experiments, batches of young leaves were picked, set up as described above and kept in the dark in an incubator at 30° C. for periods that varied in the different experiments. Distilled water only was supplied to the cut petioles. With one exception the experiments were carried out in May and June when the plants were in vigorous growth. Assays were carried out by the method of Allport & Wilson.

Table 1. *Total alkaloids in cut belladonna leaves before and after isolation*

Date	Days	Temp. (° C.)	Total alkaloids as mg. *l*-hyoscyamine in 100 g. initial fresh weight		
			Start	Finish	Difference
5 May 1944	3	30	58	56·6	−1·4
	3	30	75	68·5	−6·5
14 May 1943	3	30	37	42	5
	5½	30	37	37	0
	7	30	37	49	12
	10½	30	37	47	10
28 May 1943	5½	30	48	59	11
	8	30	48	54	6
	10½	30	48	53	5
14 June 1943	7	30	38	42	4
2 Oct. 1943	6¾	15	78	87·1	9·1
					Mean + 4·9

Statistical data: mean = +4·9, $t = 2{\cdot}84$, $n = 10$, $P = < 0{\cdot}02$.

The results indicate that under the conditions of the experiment there is a small, but statistically significant, increase in the mean alkaloid content of the leaf samples during the period of isolation. The probability of the mean increase being fortuitous is less than one in fifty. There is, however, a strong suggestion that the increase occurs only after an initial lag period, the largest increases being recorded after 5½ days, when the leaves had become ⅓–½ yellow.

Starvation experiment. An experiment was designed to examine more thoroughly the behaviour of the alkaloids during starvation. Four hundred young leaves were picked in a stage of rapid expansion. The stage selected was characterized by the lower half of the lamina being still pale green in colour, crinkled and noticeably shiny. The upper half was darker, smooth and had a matt surface. Seven lots of leaves were selected in the laboratory, each containing fifty leaves with a combined fresh weight of 23 g. One lot was dried at 50° C. for immediate analysis and each of the others was set up in the usual way in beakers containing 1 % sucrose in distilled water. They were then put into an incubator at 25° C., and one sample was removed for analysis daily for the next 6 days. Total alkaloids were estimated by the method of Roberts & James, and total, protein and soluble nitrogen by micro-Kjeldahl's. The condition of each sample was recorded as it was taken out for analysis and the solutions of the remaining samples were removed and replaced with fresh every other day.

The results of this experiment are recorded in Fig. 1. During the first 3 days there was little change in the colour, protein nitrogen and soluble nitrogen of the leaves. The small decline in alkaloid content is of doubtful significance and not observed in other experiments. After the third day an abrupt change set in. The leaves began to turn yellow, a reliable indication of protein breakdown that was confirmed by the estimation of protein nitrogen. At the same time, soluble nitrogen began to accumulate to a corresponding amount. The amount of alkaloid present increased at the same time, also. To make this clear in the figure, alkaloid nitrogen has been recorded on a scale four hundred times that of the other fractions. The increase is small; but its coincidence with the onset of protein degradation strongly suggests an origin from some fraction of the proteolysis products, perhaps some particular amino-acid or limited group of amino-acids. During the phase of increase, the alkaloids were estimated both by titration after purification (total adsorbable base) and colorimetrically (tropic acid esters). The two estimates gave good agreement (Table 2), and it is therefore safe to assume that the normal type of alkaloids were being formed (cf. pp. 173-4).

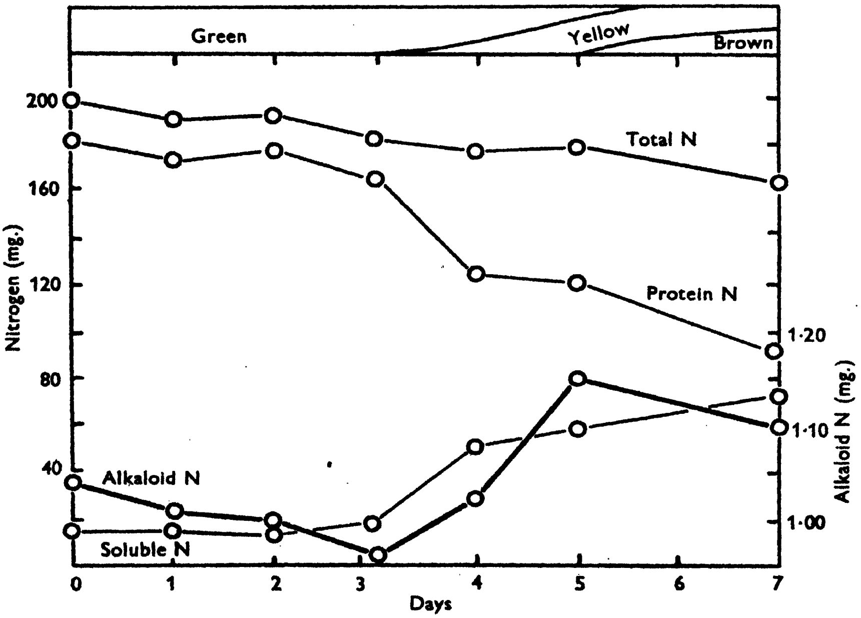

Fig. 1. Variation of nitrogen fractions in young detached leaves kept in the dark. The scale on the left refers to total, protein and soluble nitrogen. The scale on the right is four hundred times greater and refers to alkaloid nitrogen. The colour of the leaves is indicated by the strip at the top.

Table 2. *Alkaloid content of young belladonna leaves kept on* 1 % *sucrose solution in the dark at* 25° C.

	Assay by titration (% dry weight)	Assay by Vitali-Morin reaction (% dry weight)
After 5 days	0·507	0·52
After 7 days	0·505	0·52

Initial feeding experiments with L-*arginine.* A number of feeding experiments were carried out with varying results until it was realized that whatever was added, an increase of alkaloid content could only be expected in young leaves collected in a season of active vegetation. Leaves that were fully expanded or that were collected in the late autumn, rarely showed any increase. A series of experiments fulfilling these requirements was carried out with 0·20–0·25% L-arginine. Arginine was chosen because it had been suggested (Robinson, 1917) as a possible starting-point for the biological synthesis of *l*-hyoscyamine. Leaves that were fed with L-arginine in distilled water alone frequently developed signs of damage suggestive of ammonia poisoning, and when this happened the alkaloid content dropped abruptly. It was found that the damage could be minimized by feeding the arginine in a solution of 1–2% sucrose. The results obtained in six experiments are summarized in Table 3. The method of estimation was that of Allport & Wilson.

Table 3. *Synthesis of alkaloids in isolated leaves of* Atropa belladonna *fed with sucrose and* L-*arginine*

Date	Sucrose (%)	L-Arginine (%)	Days	Temp. (° C.)	Total alkaloids								
					Assay (% dry weight)			As mg. *l*-hyoscyamine per 100 g. initial fresh wt.			As mg. *l*-hyoscyamine per leaf		
					Start	Sucrose	Sucrose + L-arginine	Start	Sucrose	Sucrose + L-arginine	Start	Sucrose	Sucrose + L-arginine
5 May	2·0	0·25	3	30	0·40	0·50	0·51	58·1	65·2	68·8	0·88	0·99	1·03
5 May	2·0	0·25	3	30	0·50	0·54	0·63	74·0	74·0	89·5	1·52	1·55	1·88
5 Sept.	2·0	0·20	5	25	0·46	0·48	0·59	54·2	51·5	65·7	1·63	1·54	1·97
5 Oct.	1·0	0·20	—	25	0·34	0·46	0·41	51·9	62·6	58·1	2·15	2·59	2·41
27 Aug.	1·0	0·20	3	25	0·55	0·57	0·72	78·1	80·6	91·5	1·84	1·71	2·14
18 Sept.	1·0	0·20	3	24	0·41	0·47	0·62	63·8	61·8	83·1	1·27	1·24	1·66
Mean	—	—	—	—	0·443	0·503	0·580	63·35	65·95	76·12	1·55	1·60	1·85

The leaves used on 5 October were almost mature, growth being at that time almost finished for the season, and it is doubtful whether they should be considered as growing leaves. In all other experiments the leaves fed with L-arginine + sucrose contained more mg. of alkaloid than either those dried directly after picking or those fed on sucrose alone. Taking the results as they stand, including those of 5 October, an analysis of variance yields the following criteria.

Table 4. *Analysis of variance in feeding experiments with* L-*arginine and sucrose*

(Variate is mg. '*l*-hyoscyamine' in 100 g. initial fresh weight of leaf.)

	Sums of squares of deviations	Degrees of freedom	Variance	*z*	
				Found	For $P=0{\cdot}01$
Occasion	1821·5859	5	—	—	—
Treatment	552·7278	2	276·3639	1·280	1·0114
Remainder	213·8825	10	21·3883		
Total	2588·1962	17			

Differences between means	Difference	*t*	*P*
L-Arginine + sucrose *v.* start	12·77	4·81	$<0{\cdot}01$
L-Arginine + sucrose *v.* sucrose	10·17	3·82	$<0{\cdot}01$
Sucrose *v.* start	2·60	0·98	—

Standard error of difference of 2 means of $6=\sqrt{(\frac{1}{6}+\frac{1}{6})}\times\sqrt{21{\cdot}3883}=2{\cdot}66$.

The treatment variance is far larger than the remainder variance and, according to the '*z*' test, could arise by chance less than once in a hundred such series of experiments. The comparisons producing this large variance are those concerning the presence or absence of arginine. The estimate of the standard error of two means of six derived from the remainder variance is 2·66. This is slightly greater than the difference between the initial and the sucrose-fed samples, which is therefore of no importance. Sucrose feeding did not by itself produce an alkaloid synthesis. Both the other differences are much greater than 2·66 and, by the '*t*' test, both have *P* values below 0·01. The increases due to arginine feeding over the initial value and over the sucrose-alone value are both highly significant and indicate a definite synthesis of alkaloid, deriving from the arginine. The above analysis is based on the number of mg. alkaloid in the unit initial fresh weight. The same result would be arrived at by taking the mg. alkaloid per leaf, since the samples were chosen on each occasion to have equal numbers and equal fresh weights. Since the alkaloids were increasing at a time when the leaf carbohydrates were diminishing, their percentage in the dry weight would be expected to increase *a fortiori*, and this is shown in the column headed 'assay' (Table 3).

L-*Arginine and other amino-acids*. The above experiments showed that detached belladonna leaves might be expected to synthesize alkaloids either if they were supplied with water until their proteins began to break down, or earlier if they were fed with L-arginine + sucrose. An extensive series of experiments was planned in the following season to decide whether L-arginine was unique or whether other amino-acids would similarly act as alkaloid precursors. Ten different amino-acids were tested at a concentration of M/100 and also inorganic nitrogen in the form of ammonium sulphate. All the solutions contained 1% sucrose, including the control series without added nitrogen. A further sample of leaves was dried and analysed immediately. Estimation of the total alkaloids present was carried out by the method of Roberts & James; protein, soluble and total nitrogen were estimated by micro-Kjeldahl, as previously described. Two complete series of experiments were carried out with active young leaves, similar to—or perhaps rather younger than—those used in previous seasons. Two further series were done with even younger, still crinkled, leaves, and one with rather older leaves approaching maturity. The period of feeding was always 3 days. The whole group of experiments was carried out in the summer of 1946, between 29 May and 10 August. The results obtained with the active young leaves are given in Table 5.

The only amino-acids in these experiments that show any increased alkaloid formation over the initial and sucrose controls are those of the arginine-ornithine group. Even with these the increases are very meagre, the mean increase over the start being 12% and over the sucrose control, 21%. If the arginine and control values from this experiment are inserted into the analysis of variance on p. 177, they slightly lower the *P* values, i.e. slightly increase the significance of the increases over the start and sucrose controls due to the presence of arginine. The two further series were therefore carried out, using younger leaves, still in the crinkled and shiny stage of their unfolding. The mean initial fresh weight was 0·34 g. Treatment was identical with that in the two previous experiments. The results are not presented in full because no increases of alkaloid content were recorded with any of the amino-acids. It was thought that this might be due to a failure of the complex amino-acids to enter the cells at this stage of leaf development. Even though the volume of the feeding solution diminished, we could not be sure that the amino-acids reached the surfaces of the mesophyll cells. Recourse was therefore had to vacuum infiltration, after which the solutions were in visible contact with these surfaces and might even be driven through them.

Young leaves in the crinkled and shiny stage over the entire surface were picked from young belladonna shoots in July. Three lots of 100 leaves, graded to an initial fresh weight of 29 g., were injected with (i) 1 % sucrose, and (ii) 1 % sucrose + M/100 L-arginine. The weight after injection, washing and surface drying, was 44 g., so that 15 g. solution was taken up. The two samples were then put to incubate with the petioles dipping into water overnight and afterwards were transferred: (i) on to 1 % sucrose solution, and (ii) on to 1 % sucrose + M/100 L-arginine solution for a further 34 hr. During the latter period 18 and 16 ml. disappeared from the two feeding solutions respectively, so presumably some further solution was drawn into the leaves as the injected solutions dried out. The third sample of leaves was dried immediately after picking and sorting. Alkaloid contents were determined by the method of Allport & Wilson and nitrogen fractions by micro-Kjeldahl. The results were as shown in Table 6.

Table 5. *Alkaloid content of young, actively growing belladonna leaves fed with the indicated solutions*

(Exp. *a*, fifteen leaves in a sample. Mean initial fresh weight 2·13 g., mean initial dry weight 0·245 g., all leaves taken from a single very large plant. Exp. *b*, thirty leaves in a sample. Mean initial fresh weight 1·00 g., mean initial dry weight 0·113 g. Leaves just out of the crinkled stage, still pale green at lower end; all from a small group of related plants. All amino-acids or M/100.)

Treatment	Assay (% dry weight)		mg. per leaf									
			Dry weight		Total alkaloids as *l*-hyoscyamine		Protein N		Soluble N		Total N	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
Dried at start	0·356	0·805	278	113	0·99	0·91	14·5	6·28	1·03	0·46	15·4	6·81
Sucrose 1 %	0·330	0·997	243	99	0·80	0·99	12·0	4·95	1·53	0·88	13·4	5·88
Sucrose 1 % + ammonium sulphate	0·364	0·980	247	102	0·90	1·00	12·3	5·22	1·48	0·79	13·8	6·02
Sucrose 1 % + glycine	0·357	1·021	240	103	0·86	1·05	11·8	5·24	1·42	0·85	13·3	6·12
Sucrose 1 % + DL-alanine	0·356	0·991	242	102	0·86	1·01	12·0	5·16	1·48	0·89	13·5	6·08
Sucrose 1 % + L-valine	0·340	0·943	240	102	0·81	0·97	11·9	5·22	1·51	0·85	13·3	6·08
Sucrose 1 % + L-leucine	0·340	0·886	245	105	0·84	0·93	12·0	5·35	1·45	0·89	13·7	6·27
Sucrose 1 % + L-glutamic acid	0·279	0·946	245	98	0·69	0·93	11·6	5·01	1·69	0·75	13·5	5·81
Sucrose 1 % + L-arginine	0·440	0·982	244	103	1·07	1·01	12·0	5·22	1·52	0·91	13·6	6·14
Sucrose 1 % + L-ornithine	0·451	1·098	240	100	1·08	1·10	11·9	5·09	1·37	0·82	13·3	5·97
Sucrose 1 % + L-acetyl-ornithine	0·309	1·100	244	101	0·75	1·11	11·9	5·15	1·61	0·79	13·6	6·00
Sucrose 1 % + L-histidine	0·348	0·893	248	102	0·86	0·91	12·3	5·20	1·42	0·88	13·8	6·07
Sucrose 1 % + L-proline	0·356	0·934	249	98	0·89	0·92	12·4	5·03	1·45	0·85	13·8	5·85

Table 6. *Alkaloid contents of very young belladonna leaves*

(Mean initial fresh weight = 0·29 g.)

Treatment	Assay (% dry weight)	mg. per leaf				
		Dry weight	Total alkaloids as *l*-hyoscyamine	Protein N	Soluble N	Total N
Dried at once	0·14	52·5	0·0735	0·295	0·022	0·320
1 % sucrose	0·15	48·4	0·0726	0·249	0·023	0·275
1 % sucrose + M/100 L-arginine	0·19	49·3	0·0937	0·256	0·024	0·282

An increase in the amount of alkaloid per leaf was shown by the arginine set both over the sucrose control (29·2%) and over the initial (28·8%).

A second experiment of the same kind was carried out with the addition of a third solution containing sucrose and ammonium sulphate. Treatment was exactly as before and the leaves were in the same early stage of development. Each sample contained fifty-one leaves, with an initial fresh weight of 13 g., and the results were as in Table 7.

Table 7. *Alkaloid contents of very young belladonna leaves*

(Mean initial fresh weight = 0·255 g.)

Treatment	Assay (% dry weight)	mg. per leaf				
		Dry weight	Total alkaloids as *l*-hyoscyamine	Protein N	Soluble N	Total N
Dried at once	0·17	45·9	0·078	0·262	0·019	0·284
1% sucrose	0·24	43·6	0·104	0·268	0·023	0·292
1% sucrose + M/100 $(NH_4)_2SO_4$	0·24	42·6	0·102	0·264	0·023	0·288
1% sucrose + M/100 L-arginine	0·26	44·0	0·114	0·268	0·024	0·295

The arginine sample again shows an increase when compared with the sucrose control, but the ammonium sulphate one does not. The arginine increase is smaller than in the previous experiment, possibly due to the fact that less solution was driven in by the infiltration; only 0·059 ml. per leaf instead of 0·150 ml. The large difference between the sucrose control and the initial value is suspect, since it is not confirmed in other experiments. In these two experiments there is little or no breakdown of protein nitrogen to soluble nitrogen during the 2 days of incubation. In this respect the sucrose and arginine samples are identical, so that there are no grounds for supposing that the effect of arginine is indirect and due to any acceleration of proteolysis.

The above results suggest that L-arginine and its related amino-acid L-ornithine can increase the amount of alkaloids in belladonna leaves; they provide no indication that L-proline or any of the other amino-acids tried may do the same. As it was possible that an increase might fail to show up upon a day selected to some extent arbitrarily as in the previous experiments, it was decided to follow the alkaloid drift with time over the first 3 or 4 days of feeding. L-Ornithine and L-proline were chosen for further investigation.

L-*Ornithine*. Young, actively growing leaves were picked from belladonna bushes and divided into twelve lots, each weighing 15 g. and containing thirty-seven leaves. Two lots were dried at once; five lots were incubated on 1% sucrose and five on 1% sucrose + M/100 L-ornithine. Samples were taken for analysis by the method of Roberts & James, after 3, 4 and 5 days. The results were as shown in Table 8. The ornithine series was stopped after the fourth day, as the leaves began to turn yellow and the petioles to show damage. Similar signs appeared in the sucrose series on the fifth day. The samples giving the highest alkaloid contents, both in the sucrose only and in the ornithine series, were checked colorimetrically. Close agreement was obtained with the titration values as shown by the bracketed figures in the assay column. The increases may therefore confidently be ascribed to the usual alkaloids, and *l*-hyoscyamine was isolated as picrate from the maximal ornithine sample using the method of Trautner & Roberts (1948). The crystals obtained melted sharply at 164° C. and gave a mixed melting-point with an authentic sample of L-ornithine picrate of 164·5°.

Collecting all the ornithine experiments with young leaves gives the following results. Four comparisons were made on three occasions: in each the ornithine-fed leaves showed a small increase of alkaloid per leaf, both over the initial value and the sucrose control. Analysis of the variance gives the data shown in Table 9. The treatment variance is large compared with the remainder variance and, as computed by the z test, is likely to arise accidentally less than 1 in 20 such series of experiments. The comparisons providing the large treatment variance are those involving ornithine, both of which are highly significant ($P < 0{\cdot}05$). The presence of sucrose alone again causes no appreciable change from the start.

Table 8. *Alkaloid contents of young belladonna leaves*

(Mean initial fresh weight = 0·46 g.)

	Assay (% dry weight)		mg. per leaf			
			Dry weight		Total alkaloids as *l*-hyoscyamine	
	Sucrose	Sucrose + ornithine	Sucrose	Sucrose + ornithine	Sucrose	Sucrose + ornithine
Start	0·424		76·1		0·322	
After 3 days	0·458	0·567 (0·56)	77·4	69·9	0·354	0·396
After 4 days	0·496 (0·51)	0·558	72·0	67·7	0·356	0·378
After 5 days	0·543	—	60·8	—	0·330	—

Table 9. *Analysis of variance in feeding experiments with* L-*ornithine and sucrose*

(Variate is mg. '*l*-hyoscyamine' in 100 g. initial fresh weight of leaf.)

	Sums of squares of deviations	Degrees of freedom	Variance	z		
				Found	For $P = 0{\cdot}05$	For $P = 0{\cdot}01$
Occasion	2932·7238	3	—	—	—	—
Treatment	425·9468	2	217·9734	1·1076	0·8188	1·1955
Remainder	142·6833	6	23·7806			
Total	3501·3539	11				

Differences between means	Difference	n	t	P
L-Ornithine + sucrose *v.* start	13·9	6	4·03	< 0·01
L-Ornithine + sucrose *v.* sucrose	10·8	6	3·14	0·02
Sucrose *v.* start	3·1	6	0·90	0·4

Standard deviation = 4·876, therefore standard error of difference of 2 means of $4 = 4{\cdot}876 \times \sqrt{(\frac{1}{4} + \frac{1}{4})} = 3{\cdot}448$.

The presence of ornithine in young belladonna shoots. Young sprouts, consisting of condensed stems and young overlapping leaves, were taken from the root stocks early in the growing season. At this stage growth was rapid and alkaloid formation would be expected to be rapid also. The sprouts were homogenized with a minimal amount of water in a Waring Blendor and the proteins precipitated by immediately adding trichloracetic acid to a concentration of 5%. The mixture was brought to the boil and filtered. The filtrate was then reduced to 2 ml. by standing overnight in a vacuum desiccator over calcium chloride at room temperature. The concentrate was analysed by 'one-way' paper chromatograms according to the method of Consden, Gordon & Martin (1944). The tests were run with phenol for 24 hr. Two indicators were used, ninhydrin and a pH indicator, B.D.H. 4460. Basic amino-acids are revealed by the latter as a blue spot on a pink background, and are thereby distinguished from other amino-acids with similar R_F values. Reference strips were run with pure L-ornithine and L-arginine (Roche). Three runs with aliquots of the belladonna concentrate showed strong blue to purple ninhydrin reactions at R_F=0·39, 0·35 and 0·37 respectively. An alkaline reaction was given at the same position. The pure L-ornithine gave R_F=0·37. L-Arginine gave R_F=0·70. A strong ninhydrin reaction was given by the concentrate at R_F=0·71, 0·69 and 0·72 in the same three runs. There was, however, no alkalinity at this position and no Sakaguchi reaction could be obtained, although this is given by minute doses of arginine on filter-paper. It would therefore seem that the ninhydrin reaction at $R_F \sim$0·70 may have been due to some other amino-acid than arginine. Ornithine, on the other hand, was satisfactorily identified.

L-*Proline*. A number of experiments were carried out with L-proline in addition to those of Table 5. As the results appeared ambiguous, a time series was attempted, as with ornithine. Seven samples of thirty leaves were each selected to weigh 11·5 g. They were in the young, actively growing state with crinkled and still shiny surfaces. They were collected on 10 July and put on to the feeding solutions in separate beakers as quickly as possible after picking. Three samples were put on to 1 % sucrose and three on to 1 % sucrose + M/100 L-proline. The seventh sample was dried immediately and the others incubated at 25° C. Paired sucrose and L-proline samples were removed after 1, 2 and 4 days. The results are shown in the three bottom lines of Table 10. There is no indication of a rise in the amount of alkaloid per leaf, as with L-ornithine. The results of all experiments in which L-proline was used are collected in Table 10.

Table 10. *Alkaloid content of young, actively growing belladonna leaves fed with 1 % sucrose and* L-*proline*

Date	Method*	Proline (M)	Days	Temp. (° C.)	Assay (% dry weight)			Total alkaloids as mg. *l*-hyoscyamine per leaf		
					Start	Sucrose	Sucrose + L-proline	Start	Sucrose	Sucrose + L-proline
5 Oct.	A. W.	0·011	3	25	0·34	0·46	0·40	2·15	2·59	2·31
27 Aug.	A. W.	0·011	3	25	0·55	0·57	0·74	1·84	1·71	2·14
18 Sept.	A. W.	0·011	3	24	0·41	0·47	0·56	1·27	1·24	1·55
29 May	R. J.	0·010	3	25	0·394	0·595	0·408	0·97	1·41	0·97
12 June	R. J.	0·010	3	25	0·356	0·330	0·356	0·99	0·80	0·89
23 July	R. J.	0·010	3	25	0·805	0·997	0·934	0·91	0·99	0·92
10 July	R. J.	0·010	1	25	0·605	0·500	0·555	0·40	0·30	0·38
10 July	R. J.	0·010	2	25	0·605	0·607	0·604	0·40	0·39	0·39
10 July	R. J.	0·010	4	25	0·605	0·599	0·652	0·40	0·37	0·35

* A. W. = Allport & Wilson; R. J. = Roberts & James.

The leaves of the experiment of 29 May were probably too mature.

The results are irregular, sometimes showing a small gain with proline feeding and sometimes a small loss. Analysis of the variance shows that the small treatment contribution is much less than the remainder variance and therefore quite without significance. There is no likelihood that the proline feeding increased alkaloid formation in the leaves.

Ammonium sulphate. It might be supposed that L-arginine and L-ornithine served as alkaloid precursors merely by acting as sources of free ammonia. It has been shown (James, 1945) that ammonium sulphate supplied as a nutrient to growing belladonna plants increases alkaloid production, but this is a relatively long-term effect. Experiments were therefore carried out using the usual feeding technique, but substituting ammonium sulphate for the amino-acid. On four occasions L-arginine and ammonium sulphate were fed simultaneously to young leaves and the results are collected in Table 11.

Table 11. *Alkaloid content of young, actively growing belladonna leaves fed with* L-*arginine and ammonium sulphate*

Date	Nitrogen solution (M)	Days	Temp. (° C.)	mg. *l*-hyoscyamine per leaf			
				Start	Sucrose	Sucrose + ammonium sulphate	Sucrose + L-arginine
5 Sept.	0·011	5	25	1·63	1·54	1·68	1·97
12 June	0·010	3	25	0·99	0·80	0·90	1·07
23 July	0·010	3	25	0·91	0·99	1·00	1·01
10 July*	0·010	2	25	0·078	0·104	0·102	0·114

* Very young leaves, solutions applied by vacuum infiltration.

Table 12. *Significance of means in eleven feeding experiments with ammonium sulphate*

(Variate is mg. '*l*-hyoscyamine' in 100 g. initial fresh weight of leaf.)

Differences between means	Difference	*n*	*t*	*P*
Ammonium sulphate + sucrose *v.* start	15·46	20	4·06	< 0·01
Ammonium sulphate + sucrose *v.* sucrose	1·31	20	0·344	> 0·70
Sucrose *v.* start	14·55	20	3·82	< 0·01

Standard deviation = 8·962. Standard error of difference of 2 means of 11 = 3·818.

It is evident that ammonium sulphate does not increase the mg. alkaloid per leaf as much as arginine, if indeed it increases it at all under the conditions of these experiments. The same is true in the two comparisons available with L-ornithine (Table 5). Eleven experiments in all were carried out in which ammonium sulphate was presented to young and very young belladonna leaves by way of their transpiration stream. Analysis of the results yields the data shown in Table 12. Although the mean ammonium sulphate value is significantly greater than the mean value at the start, it is almost identical with the sucrose mean. The difference between them with $P > 0{\cdot}70$ is clearly of no significance, and there is no evidence of any increase of alkaloid content due to the ammonium ions under the conditions of these experiments. These results are all the more striking when it is remembered that ammonium sulphate probably enters the living cells much more readily than the amino-acids. It appears safe to conclude that L-arginine and L-ornithine do not owe their action as alkaloid precursors to a production of free ammonia or ammonium ions.

DISCUSSION

The foregoing experiments are taken to show that L-arginine and L-ornithine may act as precursors of tropane alkaloids in young belladonna leaves. Two possible objections may be considered. The leaves fed with L-arginine and L-ornithine yellowed a little more rapidly than the controls fed on sucrose only (cf. p. 180), and it might be supposed that the effect of the two amino-acids was only indirect and operated by a premature release of proteolysis products. A similar slight acceleration of yellowing was, however, also observable with other amino-acids that did not produce any increase of alkaloids and cannot, therefore, be regarded as its cause. Direct analysis of N-fractions did not show any greater proteolyses in the presence of arginine and ornithine than with the other solutions (cf. Tables 5–7).

In the second place, L-arginine and L-ornithine each possesses two amino-groups, free or potential, in its molecule, and so might be thought to have an advantage over the mono-amino-acids in this way alone. Ammonium sulphate and the simpler amino-acids, on the other hand, would probably be absorbed faster than the di-amino-acids with their relatively high molecular weights. As the internal concentrations could not be predicted or controlled, the amino-acids were supplied in a considerable excess over any likely conversion to alkaloid. Variations of their amino-equivalents cannot be supposed, therefore, to have had any importance.

The two amino-acids used are closely related to one another, L-arginine yielding L-ornithine + urea on hydrolysis. The enzyme arginase which catalyses the hydrolysis has been identified in belladonna shoots and in a number of related tissues (James, 1945):

$$\mathrm{H_2N.C(:NH).NH.CH_2.CH_2.CH_2.CH.NH_2 + H_2O}$$
$$\xrightarrow{\text{arginase}} \mathrm{H_2N.CH_2.CH_2.CH_2.CH.NH_2.COOH + (NH_2)_2CO.}$$

The two amino-acids may be serially related in this way during alkaloid formation. It has been shown that alkaloid synthesis can occur simultaneously with protein breakdown in our leaves, and, since L-arginine is an apparently universal constituent of plant proteins, it is likely to be the starting-point of the alkaloid synthesis. L-Ornithine is not known as a protein constituent, and probably arises only by the hydrolysis of L-arginine. Free ornithine has been identified in an extract of young, actively growing belladonna shoots. It does not necessarily follow that L-arginine is the only starting-point for alkaloid synthesis in belladonna. Negative results have been obtained with the related L-proline after numerous repetitions. Other amino-acids have also yielded negative results but, except with L-proline, the experiments have not been frequently repeated.

SUMMARY

1. Young belladonna leaves detached and kept on 1% sucrose in the dark showed an increase of mg. alkaloid per leaf beginning after 3 days. Later the alkaloids diminished. The increase was associated with loss of protein nitrogen and gain of soluble nitrogen.

2. Feeding experiments, limited to the first 3 days, were carried out with several amino-acids. M/100 L-arginine and M/100 L-ornithine supplied in addition to 1% sucrose, caused increases in the mg. alkaloid per leaf both over the initial value at the time of picking and over the value for leaves supplied with 1% sucrose only. The increases were small but statistically significant.

3. Neither M/100 L-proline nor M/100 ammonium sulphate caused similar increases.

4. It is concluded that L-arginine and L-ornithine can act as precursors of the tropane alkaloids formed in young belladonna leaves. The L-arginine may be formed by proteolysis and then be hydrolysed to L-ornithine which has been identified in the free state in young shoots.

I wish to express my thanks to Dr M. Roberts and Mrs H. M. Paterson who carried out the very numerous analyses.

REFERENCES

ALLPORT, N. L. & WILSON, E. S. (1939). A rapid colorimetric assay of Belladonna and Stramonium. *Quart. J. Pharm.* **12**, 399.

CONSDEN, R., GORDON, A. H. & MARTIN, A. J. P. (1944). Qualitative analysis of proteins: a partition chromatographic method using paper. *Biochem. J.* **38**, 224.

JAMES, W. O. (1945). *Oxford Medicinal Plants Scheme Annual Report.*

JAMES, W. O. (1946). Demonstration of alkaloids in Solanaceous meristems. *Nature, Lond.*, **158**, 377.

JAMES, W. O. & ROBERTS, M. (1945). The nature and specificity of the Vitali-Morin reaction for Solanaceous alkaloids. *Quart. J. Pharm.* **18**, 29.

PETERS, J. P. & VAN SLYKE, D. D. (1932). *Quantitative Clinical Chemistry.* London.

RICHARDS, F. J. (1934). On the use of simultaneous observations on successive leaves for the study of physiological change in relation to leaf age. *Ann. Bot.* **48**, 497.

ROBERTS, M. & JAMES, W. O. (1947). A method for the estimation of total alkaloids in Belladonna and Stramonium. *Quart. J. Pharm.* **20**, 1.

ROBINSON, R. (1917). A theory of the mechanism of phytochemical synthesis of certain alkaloids. *J. Chem. Soc.* **111**, 762, 876.

ROWSON, J. M. (1944). The assay of Belladonna and of some other Solanaceous drugs—determination of hyoscyamine, atropine and hyoscine. *Quart. J. Pharm.* **17**, 234.

TRAUTNER, E. M. & ROBERTS, M. (1948). The separation of hyoscine and hyoscyamine and the alkaloidal assay of *Duboisia* spp. *Analyst*, **73**, 140.

WATSON, G. M., HEARD, C. R. C. & JAMES, W. O. (1941). A comparison of the biological and chemical assays of Belladonna and Stramonium. *Quart. J. Pharm.* **14**, 253.

WEEVERS, TH. (1933). Die Pflanzenalkaloide. *Rec. Trav. bot. néerland.* **30**, 336.

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STUDIES IN STOMATAL BEHAVIOUR

II. THE ROLE OF STARCH IN THE LIGHT RESPONSE OF STOMATA. PART 1. REVIEW OF LITERATURE, AND EXPERIMENTS ON THE RELATION BETWEEN APERTURE AND STARCH CONTENT IN THE STOMATA OF *PELARGONIUM ZONALE*

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(With Plates 1–10)

INTRODUCTION

Even a cursory survey of the literature dealing with stomatal behaviour leads to the conclusion that despite a great deal of work over a long period of time the whole matter remains obscure. This may perhaps be attributed to two causes: (1) the difficulties encountered in observation of stomatal aperture and its changes; (2) the naïve assumption that stomatal movement is regulated by a single mechanism, in face of the fact that the response of stomata to at least six stimuli appears well established. These are (*a*) light and darkness, (*b*) changes in water content of the leaf, (*c*) temperature, (*d*) CO_2 content of the air, (*e*) shock stimuli, (*f*) pH changes, (*g*) ionic effects. The adequacy of postulated mechanisms will in the final event need to be tested by a thorough investigation of the dynamics of the opening and closing reactions. The aim of this series of papers is to widen our knowledge of stomatal behaviour and to provide data for the postulation of the underlying mechanisms, bearing in mind the variety of the effective stimuli. In the first paper (Williams, 1948) an investigation of stomatal response to heat-shock stimuli is described. In this paper the problem of the light reaction is considered in relation to the possible role of the starch $\rightleftharpoons$ sugar balance in the stomatal apparatus.

Dating from some of the earliest investigations on stomata (von Mohl, 1856; Schwendener, 1881) the movements of the guard cells in response to light and other stimuli have been very generally attributed to turgor changes. Von Mohl showed that stomata could be made to open or close by immersion of epidermis in water or sugar solutions. Schwendener's measurements showed that when stomata are closed by immersion in plasmolysing solutions the guard cells are reduced in volume. A number of investigators (Iljin, 1914; Wiggans, 1921; Steinberger, 1922; Sayre, 1926; Strugger & Weber, 1926) have found by plasmolysis methods that the osmotic pressure of the sap of the guard cells rises with diurnal stomatal opening, while that of the epidermal cell sap changes little, if at all. The smallest ascertained change between closed and wide open stomata is that found by Sayre (1926) for *Rumex patientia*, and even this amounts to about 6 atmospheres. If entirely caused by sucrose this would mean an approximate increase in sucrose concentration by 8½%; for glucose the corresponding figure would be 4½%.[*] Although plasmolytic methods present many pitfalls, it appears evident that considerable increases in osmotic pressure do occur with normal stomatal opening. These must be accompanied by increased turgor of the guard cells and would therefore provide the force necessary for opening. Recent work (Heath, 1938) has shown that in *Cyclamen* and *Tradescantia* a 'wall mechanism' provides no appreciable part of the motive force in stomatal opening, and that a turgor mechanism alone is concerned. The question arises as to how the necessary changes in turgor are produced in response to light or darkness. A careful examination of some of the relevant data published from 1898 onwards suggests that the answer is still highly problematical.

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[*] This neglects changes in the volume of the vacuole.

The common occurrence of starch in the plastids of stomatal guard cells, even under conditions in which it was absent from the rest of the leaf, led very early to great importance being attached to this substance as a factor in stomatal movement. Thus Kohl (1895, as cited by Lloyd, 1908, p. 130) considered that: 'The fact that the guard cells always contain an abundance of starch indicates that this plays an important role. The ever-present chlorophyll bodies produce in the course of their development a continually increasing amount of starch, which is quickly changed under appropriate stimuli into powerfully osmotic substances.' He was unable to determine what these substances were, but found that stomatal movement occurred only in response to the same regions of the visible spectrum (red and blue) as were most effective in photosynthesis. (He also found stomatal opening due to dark heat rays.) He concluded (quoted in German by Lloyd, 1908): 'As the increase in turgor of the guard cells which leads to opening occurs in these parts of the spectrum, I conclude that in the first place the production of starch in the chloroplasts of the guard cells as well as the starch-hydrolysing enzymes in the guard cells are affected.'

Lloyd (1908), working mainly with a desert plant, *Verbena ciliata*, found generally a diurnal rhythm in the starch contents of the guard cells, which he claimed was correlated with stomatal movement. After sunrise, the starch content fell to a minimum between 8 and 11 a.m., but rose again during the afternoon, stomatal opening following approximately the converse course. His other experiments in which conditions were artificially controlled show much less correlation between starch content and stomatal aperture, and these will be considered further in the Discussion. In one experiment, after a shoot had been exposed during six days to blue light, the guard cells were found to remain devoid of starch but the stomata were still motile. Lloyd claimed that with progressive starvation of the leaf and depletion of the starch in the guard cells the maximum opening was slowly reduced; but his fig. 39 (p. 114) gives little evidence of this, and since the night closure became more complete as the experiment proceeded the *range* of movement actually increased as the stomata lost their starch. Lloyd considered the possibility that this opening might have been due to the production of osmotically-active substances by photosynthesis in the plastids of the guard cells, but in general he held that such photosynthesis was relatively unimportant, and that the main cause of stomatal opening was an enzymatic digestion of starch. Closure during the afternoon was, he thought, due to withdrawal of osmotic substances by reformation of starch and by outward diffusion, and these processes could also occur in the dark.

Rosing (1908) found in *Impatiens Balsamina* a marked decrease in the starch content of the guard cells of stomata opening in the light during the morning. The plant was then placed in the dark, at 12.30 p.m. Stomatal closure followed and was accompanied by an increase of starch in the guard cells. Here a diurnal rhythm in starch content might have been the cause. Tests with Fehling's solution showed reciprocal changes in the amounts of reducing substances in the guard cells, but Rosing does not appear to have been satisfied as to the nature of these substances nor that the technique was reliable. A further experiment with *I. parviflora* gave similar results for stomatal apertures and starch, and in this case comparison with a control plant kept in daylight and having open stomata throughout showed no marked differences in starch content between the two, which would seem to indicate that a diurnal rhythm was in fact concerned. Here the 'reducing sugar' content of the guard cells was apparently unaffected by light or stomatal opening.

Iljin (1914), in a paper frequently cited as evidence for the disappearance of starch with stomatal opening (a light response being implied), refers only to the effects of atmospheric humidity and wilting, and all his experiments were carried out in the light. Using *Centaurea orientalis*, *Origanum vulgare* and *Campanula glomerata* he found that conditions of high evaporating power led to stomatal closure and an increase of starch. Wilting of a detached shoot of *Origanum vulgare* was accompanied within half an hour of cutting by the accumulation of large quantities of starch in the guard cells.

Hagen (1918) found marked diurnal changes in stomatal starch content and aperture in *Tradescantia virginica*, similar to those found by Lloyd for *Verbena ciliata*. He also found that injection of the intercellular spaces with diastase solution caused the stomata of *Peperomia marmorata* to open widely and their starch content to decrease noticeably after 24 hr. in the solution.

Loftfield (1921), in an extensive investigation, found diurnal changes in the starch content of the guard cells, again very similar to those of Lloyd, using Lombardy poplar, alfalfa and *Rumex patientia*. In the two last the correlation with stomatal movement was, however, much less complete, largely owing to midday closure and subsequent opening, and in connexion with his alfalfa observations he states: 'Hence, at times there existed a good correlation between the changes in starch content and stomatal movement and at other times none whatever. It seems clear that the entire subject is more complex than either Iljin or Lloyd has supposed, and must be the subject of renewed investigation.'

Sayre (1923, 1926), using *R. patientia*, found a similar diurnal rhythm in stomatal starch content and also an inverse change in reducing sugars. He estimated that the latter were 2 to 2½ times as abundant in stomata open in the morning as in those closed at night. Correlated with these changes were the variations in osmotic pressure mentioned earlier, and it will be seen that in terms of glucose a change from about 4½ to about 9% concentration in the cell sap is implied. The correlation of stomatal movement with these changes was again somewhat incomplete. Morning opening and evening closure only approximately reflected changes in carbohydrate and osmotic pressure, while midday closure, which was found in many cases, and the subsequent afternoon opening were independent of such changes. Midday closure is attributed, both by Loftfield and by Sayre, to water loss rather than to light, but in this connexion the increase of stomatal starch which normally accompanies wilting (cf. Iljin, cited above) should be remembered. Loftfield himself, on the assumption that the midday closure is a response to water loss, calls attention to the discrepancy between his results and those of Iljin. It may be noted here that Nutman (1937) showed that in *Coffea arabica* midday closure is brought about by high light intensity in the absence of water-strain; no observations of stomatal starch were made.

Sayre's pioneer investigations of the effects of pH changes on the guard cell mechanism will be considered more fully in the Discussion, but a brief outline of his hypothesis, which was later adopted by Scarth, Small and others, should be given here. The effect of light, according to Sayre's hypothesis, is to bring about a decrease in the acidity of the guard cells, either by oxidation of organic acids to carbon dioxide and water, by 'some rearrangement of the components of protoplasm in light where acids are decreased', or by the removal of carbon dioxide of respiration by photosynthesis. Later workers have preferred the last suggestion. This decrease in acidity increases the hydrolytic activity of diastase, starch is converted into sugar, the osmotic pressure in the guard cells rises and the stoma opens.

Strugger & Weber (1926) claim that in *Galium mollugo* changes in stomatal starch are accompanied by converse changes in the epidermal and especially in the subsidiary cells. They state that diurnal changes were first followed, and then the changes produced by darkening and illuminating the leaves; unfortunately they give no information as to the time of day when the latter experiments were carried out. No data are presented, and their figures appear to be idealized diagrams for they show open stomata with the starch grains stained in iodine-chloral hydrate, which has been found to produce closure. They characterize the normal and most frequent type of stomatal behaviour as follows: (1) With completely closed stomata, guard cells contain much starch but the subsidiary cells and epidermal cells none or a trace only. (2) With maximal opening which has been maintained for a long time the guard cells have no starch or a trace, the subsidiary cells very much starch and the epidermal cells much starch. (3) Between the extremes noted, when the aperture is of medium dimensions or has been wide for a short time only there are all imaginable variations in starch content which make the analysis of the actual relationships very much more difficult. This last state of affairs is typical of the present writer's experiences in attempting to judge starch contents of stomata. From this they deduce that the subsidiary and epidermal cells play a part in stomatal regulation and it is stated that during closure the osmotic pressure of these cells rises considerably above that of the guard cells. The stomatal apertures in *G. mollugo* are said to change very rapidly when illuminated under natural conditions, and this, together with the description of the 'normal' behaviour cited above, suggests that changes in stomatal starch if anything follow rather than precede the changes in aperture. How greatly the condition of the stomata may vary from this 'normal' state of affairs is indicated by the statement that it was often noted at night between 9.30 and 11.30 p.m. that with completely closed stomata the starch in the guard cells was entirely absent. (Alexandrov (1925) also found a starch minimum in the guard cells of *Portulaca* at midnight.) Strugger & Weber (1926) suggest that a consideration of such 'abnormal' cases is made possible by the connexion between the reciprocal regulatory systems of the guard cells and subsidiary plus epidermal cells.

Scarth (1932), who worked mainly with *Tradescantia zebrina*, states in his summary: 'All major stomatal movements in the plants studied, whether due to changes of illumination, of leaf turgor, or of temperature, are accompanied by reversible transformation of carbohydrate...', but he gives no results for the effect of *light* on starch changes in the guard cells and no data at all for sugar; furthermore, in an earlier paper (1929), he presents data for change of aperture with pH in the complete absence of starch.

The investigations just summarized have led to a very general acceptance of the hypothesis that light-induced stomatal movement is due to turgor changes brought about by a starch ⇄ sugar mechanism. Scarth (1929) has suggested that such a mechani would be too slow to account for the rapid initial light response, but he accepts it supplying the main motive force.

Recently Small and his collaborators (Small & Maxwell, 1939; Small, Clarke Crosbie-Baird, 1942) have confirmed for a wide range of species the findings of Say (1926) and of Scarth (1929, 1932) that stomata in the light are less acid than those in t dark, and they have estimated the approximate natural pH ranges by means of indicato They have also observed in detached strips of epidermis the changes in aperture and starch content of the guard cells brought about by immersion in buffer solutions at different pH values. Marks (from 0 to 2 or 3) were allotted for aperture and starch content as estimated by inspection and 'average' values so obtained. As was found by Sayre for *Rumex patientia* (1926) and by Scarth for *Tradescantia zebrina* (1929, 1932), the aperture changed with pH of the external solution and starch content was on the whole negatively correlated with aperture. Their data for both apertures and starch contents fluctuate violently and irregularly with pH, generally with two or more maxima and minima, and completely different results are found with different buffer mixtures. They again put forward the starch ⇄ sugar hypothesis of stomatal movement in the form originally postulated by Sayre (1926) except that in their last paper (Small *et al.* 1942) they apparently attribute to phosphorylase the role previously ascribed to one or more unspecified amylases. In this last paper they show that the action of diastase on starch *in vitro* may be rapid, thus throwing doubt on Scarth's objection mentioned above, and they make the sweeping claim: 'The solution of the main problems concerning the mechanism of stomatal movements is completed by the data presented above.' It should be noted, however, that no direct experiments on the effect of light on the starch content of the guard cells were carried out in their work. Furthermore, experiments on detached strips of epidermis are not convincing unless it can be shown that stomata on such strips will respond to light in the normal way. So far as the author is aware this has not been shown in any published work, and in his own experiments no such response has yet been obtained. Semmens (1947) claims that, both in the entire leaf and in detached epidermis of several species, *polarized* light causes disappearance of starch from the guard cells and opening of the stomata, whereas with normal daylight they remain closed and full of starch. This work is considered further in the Discussion, but in any case such behaviour can scarcely be considered a *normal* response to light. It may be that the control of stomatal opening by light cannot occur in the absence of the mesophyll solely because it depends upon the assimilation of carbon dioxide by the chlorophyllous tissues; it should then be possible to cause stomatal movement in detached strips of epidermis by altering the carbon dioxide tension in the surrounding liquid. This also the author has so far failed to achieve. It is hoped to consider the question of stomatal control by carbon dioxide in later papers in this series; a preliminary communication on the subject has already been published (Heath, 1948).

The criticism that stomata on detached strips of epidermis fail to respond to light would appear to apply also to the use of surface sections (cf. Arends, 1926), although Scarth (1932, p. 490) implies that stomata on thick sections of *Zebrina* leaf will open in the light. This criticism, if well founded, applies to many of the numerous investigations of the effects of salts on stomatal aperture and starch content (e.g. Arends, 1926), but these investigations are not relevant to the problem of light response under consideration here.

It will be noted that nearly all the direct evidence for an effect of light on starch content of the guard cells is derived from the correlation of diurnal trends, an unsafe basis for a causal theory, and even these correlations, as will be seen in later examination of the work of Lloyd and of Sayre, are not always satisfactory.

The demonstration that, even in a single species, the usual type of stomatal response to light *normally* occurs in the absence of starch, would disprove the generality of the hypothesis and throw some doubt on its validity as applied to other plants. If it is further shown that, in species which normally have starch in the guard cells, responses to light can be induced in the absence of starch, as was achieved by Lloyd in the blue-light experiment on *Verbena* cited above,* or without appreciable changes of starch content, the hypothesis must rank as of secondary importance at most and a different mechanism must be sought. Of course the hydrolysis of some soluble polysaccharide can always be postulated, but evidence for this is desirable if it is to be made a working hypothesis.

Parkin (1899) was unable to find either starch or chloroplasts in the guard cells of the onion leaf. He was also unable to induce the formation of starch in onion stomata by floating pieces of leaf on a 2% sucrose solution. The question therefore arises as to whether the stomata are motile and if so whether they respond to light in the usual manner. That they are motile is shown by the work of Loftfield (1921) who took strips of leaf epidermis for examination by Lloyd's (1908) method at hourly intervals throughout a 24 hr. period. Estimates of stomatal aperture obtained by Lloyd's method are subject to great variation unless a large number of stomata is measured for each occasion, and further there is the likelihood of systematic errors if special precautions are not taken to ensure unbiased sampling. Small fluctuations found by this method, such as the night opening found by Loftfield for the onion (see his fig. 20), may perhaps be discounted, but the main trends of his graph almost certainly represent real changes in mean aperture, including probably the considerable degree of closure shown at midday. Work by Ashby (1931), Newton (1936) and the author (unpublished) comparing stomatal apertures as estimated by Lloyd's method with porometer results, has shown that increases in mean aperture found by the former method do reflect real increases in aperture on the living leaf and this is all that is necessary for the present argument. Both Loftfield's fig. 20 and his photographic illustrations (his plate 8) are strongly suggestive of a response to light. His records, however, give only the march of stomatal movement over 24 hr. with none of the external factors controlled. The possibility had, therefore, to be considered that an autonomous diurnal fluctuation, such as those found by Maskell (1928) for cherry laurel and by Gregory & Pearse (1937) for *Pelargonium* under constant illumination, was concerned rather than a light effect. Further possibilities were that the stomatal opening was due to factors other than light, viz. air temperature, relative humidity, soil moisture or even perhaps the radiant heat asssociated with insolation (cf. Kohl cited above). Experiments to be described in the next paper of this series have shown that none of these factors accounts for the stomatal opening in the onion and that there is a real response to light. In describing stomatal movement in general Loftfield (1921) says (p. 75): 'Light induces opening of the stomata after daybreak by initiating conversion of the starch within the guard cells into sugar....' He draws no attention to the absence of starch in the guard cells of the onion. It is noteworthy that in Maximov's text-book

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* The possibility of the opening being due to a diurnal rhythm rather than a light effect cannot be excluded.

(1938) Loftfield's excellent photographs of onion stomata are the only illustrations of such structures, except for three small diagrams of an unnamed species.* A description of the usual starch ⇄ sugar mechanism is given although prefaced by the remark that: 'The chemical processes causing the changes in turgor of the guard cells and thus controlling stomatal movements are very peculiar and not yet fully understood in all their details.'

Hagen (1918) found no starch in the guard cells of *Allium cepa*, *A. schoenoprasum* or *A. porrum*; 'glucose' (i.e. reducing substance) was found in the stomata of the last named.

Like Hagen, Steinberger (1922) found the guard cells completely starch-free in *Allium schoenoprasum*, *A. ursinum* and *A. porrum*. She states, without presenting data, that the stomata show normal motility, and that the osmotic pressures are higher in open than in closed stomata.

A preliminary examination of *Pelargonium zonale* leaves was made in an attempt to induce light-operated stomatal movement without correlated changes in stomatal starch in a plant normally having starch in the guard cells. Long experience in this Institute had shown the stomata of this plant to be extremely responsive to light and reasonably easy to examine by Lloyd's method. It was at once discovered that no very obvious differences in starch content existed between stomata normally open in the light and those closed in the dark at the same time of day. Experiments (pp. 195–8) on the effects of light and dark at the same time of day were carried out, to eliminate the possibility of a diurnal rhythm being concerned. Furthermore, advantage was taken of material collected in 1938–9 in the attempted calibration (unpublished) of the Resistance Porometer (Gregory & Pearse, 1934; Heath, 1941) in terms of stomatal aperture as estimated by Lloyd's method. This material consisted of strips of *Pelargonium* epidermis, each taken from within the porometer cup area immediately after a reading, for which the stomatal apertures had been measured (means of 60 stomata per strip). All that was needed was to examine them for starch, and for this purpose a new reagent was found (Heath, 1947) which did not cause closure of the 'fixed' stomata as in the usual test with aqueous iodine and potassium iodide used by Lloyd, Loftfield, Sayre, Small and others. It was thus possible to observe the aperture and starch content simultaneously in the same individual stomata which Lloyd, for instance, had been unable to accomplish. His observations of starch and aperture in the same stoma were limited to one or two examples where he added the reagent while the stoma was in the field of the microscope.

In the present paper experiments are described dealing with stomatal aperture in relation to starch content after varying periods of illumination, as seen in strips of *Pelargonium* epidermis taken by Lloyd's method. Since no quantitative measure of starch is available, photographs are presented. In this section of the work it would be most desirable to include a repetition of Lloyd's experiments with blue light mentioned above. An attempt is being made to obtain seed of the *Verbena ciliata* used by Lloyd from America, but as considerable time must elapse, even after the seed is obtained, before the experiment can be made it seems undesirable to hold up publication of the rest of the work. Attempted experiments with blue light on *Pelargonium zonale* and *Impatiens parviflora* failed to prevent all starch synthesis or to remove all starch from the guard cells before death of the leaves ensued. They will not therefore be described in detail.

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* These diagrams, which Maximov labels 'Changes in starch content of guard cells during their movement (after Iljin)' are actually taken from Weber (1923) and illustrate the changes in starch content and stomatal aperture in *Impatiens parviflora* on immersion in M/10 KCl, KCl + $CaCl_2$ and $CaCl_2$ respectively.

In the subsequent paper, porometer experiments with onion leaves to discover whether the stomata are motile and respond to light in the usual manner, will be presented.

EXPERIMENTAL*

(*a*) TECHNIQUE

Stomatal apertures. Both stomatal aperture and starch content were observed in strips of lower epidermis taken by Lloyd's (1908) method. In order that the strips might be removed and plunged into absolute alcohol as rapidly as possible after making an incision, a pair of curved entomological forceps was used in which one blade had been ground to a sharp knife edge and the other adjusted so as to close exactly parallel with it. These forceps enabled the incision to be made, the cut edge of the epidermis siezed and a strip removed into alcohol with two rapid and almost continuous motions. The method has been found reliable for *Pelargonium* in that camera lucida drawings of the strips and the areas from which they are taken show that no appreciable change in area of the epidermis occurs on stripping; furthermore, changes in aperture as seen in such strips do reflect real changes in aperture on the living leaf as indicated by porometer readings. The actual relationships of stomatal aperture from Lloyd's strips and stomatal resistance from porometer readings are not concerned here. The strips were stored in absolute alcohol in sealed specimen tubes and examined in the same medium; under these conditions it has been found that no significant change in stomatal aperture can be detected even when the measurements are repeated after several years.

In the 1938–9 experiments (section *c* below) mean dimensions of the stomatal apertures were obtained by measuring a sample of stomata, usually 60, on each strip. A series of parallel traverses was made with the mechanical stage and all stomata which passed under the central cross-wires of the eyepiece micrometer were measured, unless damaged or tilted out of a plane at right angles to the optic axis. In the 1944–5 experiments (section *b* below) no micrometer measurements were made and the stomatal apertures were compared by inspection only. This was done first on the actual strips and then, in Exps. 12 and 30, on low-power photomicrographs some of which are reproduced here.

Stomatal starch content. In the earlier experiments of 1944 (Exps. 8 and 9) starch was stained with the usual iodine-potassium iodide reagent, using the technique as described by Loftfield (1921), except that ordinary distilled water was used for washing instead of 'slightly alkaline water'. The iodine solution, being aqueous, causes closure of the stomata in the Lloyd's strips and it is thus impossible to judge simultaneously and in the same stomata both starch content and aperture. Various other methods of staining the starch grains were therefore tried, including iodine dissolved in phenol as recommended by Kisser (1930) for starch in sections of tissues. Kisser notes that for starch coloration with iodine alone it is essential that the grains should be in an imbibed condition or that the reagent should contain a certain quantity of water and this has been confirmed by the present writer. Kisser recommends various mixtures of iodine in phenol with iodine in chloral hydrate, alcohol or glycerine, so that water may be added without forming emulsions. In the present experiments, iodine was dissolved in the solution of water in phenol formed when pure phenol deliquesces in air at room temperature. The solution thus contained the minimal quantity of water necessary to liquefy the phenol. Strips of epidermis transferred direct from absolute alcohol to this iodine-phenol reagent showed no stomatal closure and the contrast was excellent owing to the high refractive index (1·55 for pure phenol). The nucleus and cytoplasm were clearly visible, but the starch grains stained a rather pale yellow-brown and were not well differentiated. It was then found that the addition of an excess of potassium iodide to the iodine-phenol solution very greatly increased the differentiation of the starch grains which now stained a deep reddish brown. The nucleus and cytoplasm were much less visible than in the absence of potassium iodide, but the form of the starch grains and cell walls was clearer owing to the even higher refractive index of the new reagent (the refractive index of pure solid potassium iodide is 1·68). The chloroplasts were quite invisible but their position and shape could be estimated from the arrangement of the enclosed starch grains. In connexion with the increased staining of the starch grains in the presence of potassium iodide, the theory that potassium tri-iodide is adsorbed by the starch may be mentioned (Angelescu & Mirescu, 1928).

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* All times are given in G.M.T.

The formula finally adopted for the iodine-phenol-potassium iodide reagent was: solution of water in phenol, 100 c.c. (minimal water concentration for liquid state); iodine, 2·5 g.; potassium iodide, excess.

Staining of the starch occurs in a few minutes and the material may be mounted in the reagent for examination or photography. If mounted in pure phenol with minimal water content, no fading occurs in several hours, but the definition is somewhat impaired by the lower refractive index.

The reagent was found to cause no stomatal closure, even in strips transferred to it after only 5 days in absolute alcohol.

The iodine-phenol-potassium iodide reagent was used for staining stomatal starch in strips of *Pelargonium* epidermis from the two series of experiments described in sections *b* and *c* below. No quantitative measure of stomatal starch was available and comparison by visual inspection was therefore supplemented by comparison of photomicrographs. The method of selection of the stomata to be photographed was thus a matter of great importance. In the case of low-power photomicrographs (sections *b* and *c*), intended to give a general impression of the mean aperture and starch content, that field of view was selected which gave the greatest number of stomata in the same plane of focus. In this way subjective bias in the matter of aperture and starch content should have been avoided. For high-power photomicrographs (section *c* only), in strips from illuminated leaves, wide open stomata were deliberately selected, and of these the two having maximal and minimal starch respectively were photographed. Similarly, pairs of closed or almost closed stomata were photographed on strips from darkened leaves. These pairs of high-power photomicrographs showing maximal and minimal starch for a given aperture are presented in order to show the *range* of starch content, while some idea of the mean starch content may be gathered from the low-power photomicrographs. Some of the latter for experiments in section *c* are omitted when a fair idea of the mean starch content may be ascertained from the high-power photomicrographs showing maximal and minimal starch. It may here be noted that in open stomata the chloroplasts tend to lie in different planes of focus to a much greater extent than in closed stomata. For this reason the starch content of the open stomata tends to be underestimated from the high-power photomicrographs.

(*b*) EXPERIMENTS WITH DIURNAL RHYTHMS ELIMINATED, 1944–5

In these experiments observations were made on strips of epidermis taken from a selected leaf on each of two differently treated plants at the same time of day, any effects of diurnal rhythms either in stomatal aperture or starch content thus being eliminated. Potted plants of *P. zonale* 'Clone 5' (Heath, 1941) were used in all the experiments. Fully expanded leaves of more than 3 in. diameter, free from symptoms of senescence, disease or insect damage, were carefully matched in respect of colour, size and position on the plant (' paired leaves').

Experiment 8 (5–6 *June* 1944)

One plant was placed in a dark shed and the other in an unshaded frame at 3 p.m. on 5 June 1944; epidermal strips were taken the next day at 10.10 and 10.20 a.m. respectively.

Results

Examination of the strips mounted in absolute alcohol showed: *light*—all stomata moderately open; *dark*—most stomata slightly open, a few closed, average aperture definitely less than in light. The starch was then stained with aqueous iodine-KI. Nearly all the stomata closed completely. On the strips from the light the guard cells contained large starch grains and the chloroplasts in the epidermal cells also contained starch. The strips from the dark showed smaller, more scattered and less numerous starch grains in the guard cells, and none in the epidermal cells.

Experiment 9 (6–7 *June* 1944)

As in the previous experiment, two plants were placed in the dark shed and unshaded frame respectively at 3 p.m., 6 June 1944. Epidermal strips were taken at 10.10 and 10.20 a.m. on 7 June 1944, and the positions of the plants were then reversed. Further strips were taken at 3.20 p.m. from the illuminated leaf and at 3.50 p.m. from that in the dark. The same two leaves were used as at 10.10–10.20 a.m. The sky had been heavily overcast all day with little change in light intensity. At 10.0 a.m. the relative light intensity in the frame was 150 units, as measured by a photoelectric meter; at 3.15 p.m. it was 130 units.

Results

(1) *Stomatal aperture*, 10.10–10.20 a.m. The strips from plant A (illuminated) showed the stomata practically all wide open. Those from plant B (dark) showed some closed stomata but most were moderately open.

3.20–3.50 p.m. Plant B (light): some stomata closed, most moderately open and a few wide open. Plant A (dark): some stomata closed, most moderately open. Average opening probably less than in B (light) but much less difference than at 10.10–10.20 a.m.

(2) *Stomatal starch content*, 10.10–10.20 a.m. Plant A (light): variable, but some stomata with all the chloroplasts much distended by large starch grains. Starch in epidermal cells. Plant B (dark): starch grains very small and in fewer of the chloroplasts. Maximum, minimum and average starch contents all definitely less than in A. (Confirmed by three independent observers who were unaware of the treatments.) No starch in epidermal cells.

3.20–3.50 p.m. Plant B (light): little change in the guard cells as compared with 10.20 a.m., but if anything an increase in starch; starch had appeared in the epidermal cells. Plant A (dark): little change in guard cells as compared with 10.10 a.m. Still definitely more starch than in B (light). Starch still present in epidermal cells.

The results of this experiment thus made it clear that differences in stomatal starch between individual leaves might greatly exceed those due to light and dark treatments, unless a most improbable interaction between light and time of day were postulated; the most important comparisons to make were therefore those between strips from the same leaf at the same time on consecutive days with different light or dark treatment.

Experiment 12 (12–14 *June* 1944)

/ As before, two leaves were used, one on each of two plants A and B. Up to 10 a.m. on 12 June 1944 the two plants were in a cool frame under light 'summer cloud' shading. B was then placed in a lagged cupboard in the dark for 24 hr. A was given 24 hr. illumination: it was left in the frame until 5.45 p.m. and then placed overnight at a distance of 10 in. from a 200 W. lamp, with water screen. It was replaced in the frame at 7.30 a.m. on 13 June 1944. After the first 24 hr., at 10.5 and 10.10 a.m., epidermal strips were taken of A and B respectively, and the plants were interchanged. During the second 24 hr. period, while A was in darkness, B was given artificial light as above from 6.18 p.m. to 7.30 a.m. and was in the frame for the rest of the time. Further strips were taken at 10.5 and 10.10 a.m. on 14 June 1944 at the termination of the experiment. Some data for relative light intensity and temperature are shown in Table 1.

Table 1. *Experiment* 12 (12–14 *June* 1944). *Light intensities and temperatures*

Date	Time	Plant A				Plant B			
		Rel. light intensity	Temp. (° C.)	Max. temp. (° C.)	Min. temp. (° C.)	Rel. light intensity	Temp. (° C.)	Max. temp. (° C.)	Min. temp. (° C.)
12. vi. 44	10.0 a.m.	> 500		24	16.	0			
	11.15 a.m.	> 500							
	4.0 p.m.	100							
	5.45 p.m.	40							
	5.48 p.m.	40	24					16	14
13. vi. 44	7.30 a.m.		18						
	9.20 a.m.	60	15						
	10.10 a.m.	0		16	14	100	18	34	18
	10.30 a.m.					100–500	21		
	11.25 a.m.					> 500	29		
	6.15 p.m.					30	21		
	6.18 p.m.					40	22	22	15
14. vi. 44	7.30 a.m.						18		
	9.15 a.m.					60–> 500	25		
	10.10 a.m.						22		

Results

(1) *Stomatal aperture.* Both in A and B the stomata were definitely much less open after 24 hr. in darkness than in light, although this difference was less marked in B than A (compare Pls. 2–3, figs. 3, 4; 2, 1).

(2) *Stomatal starch content.* In spite of prolonged and careful examination it was not found possible to detect any consistent differences in this respect. This was confirmed by two independent observers who were unaware of the treatments, except that one thought that the 'A-Dark' strip showed slightly *less* stomatal starch than the other three. The starch contents may be judged from figs. 1–4 in Pls. 2–3.

Experiment 30 (25–29 *June* 1945)

As in the previously described experiment, 'paired' leaves on two plants, A and B, were used. The plants were given alternate 24 hr. periods either in complete darkness (within a ventilated cardboard shade painted white on the outside) or exposed to the natural alternation of day and night. The plants stood side by side in a greenhouse, with the blind lowered during sunny periods. At the end of each 24 hr. period, at about 10.30 a.m., strips were taken from each of the two leaves and the plants interchanged. The temperatures were somewhat higher in the dark shade than outside (Table 2) and the CO_2 content and humidity must also have been higher. Plant A was found wilted at 5.35 p.m. on 26 June 1945 but recovered on being watered and was quite turgid when strips were taken the next morning.

Table 2. *Experiment* 30 (25–29 *June* 1945). *Light and temperature*

Date	Time	Plant A		Plant B	
		Temp. (° C.)	Light	Temp. (° C.)	Light
25. vi. 45	10.44 a.m.	28	Nil	—	Sun
	12.39 p.m.	30	Nil	28	Sun
	1.45 p.m.	30	Nil	28	Sun
	3.35 p.m.	29	Nil	27	Sun
	5.45 p.m.	25	Nil	24	Sun
26. vi. 45	10.10 a.m.	19	Nil	19	Cloud
	10.20 a.m.	20	Cloud	20	Nil
	5.35 p.m.	19	Cloud	19	Nil
27. vi. 45	10.5 a.m.	15	Cloud	15	Nil
	10.35 a.m.	18	Nil	17	Cloud
	1.5 p.m.	27	Nil	24	Sun
	2.54 p.m.	29	Nil	22	Sun
28. vi. 45	10.40 a.m.	17	Nil	15	Cloud
	2.50 p.m.	14	Cloud	14	Nil
29. vi. 45	10.40 a.m.	17	Cloud	19	Nil

Results

(1) *Stomatal aperture.* The leaf of plant B showed very little stomatal opening, the apertures varying similarly from complete closure to slight opening on all strips irrespective of the light treatment. In the leaf of A, the average aperture, which was small after the first 24 hr. in darkness, showed a further decrease after the next 24 hr. with illumination, possibly as an after-effect of wilting. The third 24 hr., in darkness, produced opening to an aperture if anything rather larger than that at the end of the initial period, and the fourth 24 hr. with light produced a further opening (Pl. 3, figs. 5, 6). This further opening in the last 24 hr. was presumably a light effect.

(2) *Stomatal starch content.* B showed if anything a slightly lower starch content after each of the dark periods than after the preceding 24 hr. periods with light. A had a medium average starch content after the first 24 hr. in the dark, and this showed if

anything a slight increase in the next 24 hr. in the light, possibly as a result of wilting. The third 24 hr. in the dark was accompanied by a distinct decrease in stomatal starch and the final period with illumination caused no further change. Thus, although there was some suggestion of an effect of CO_2 or humidity on stomatal starch, there was no indication of a reduction due to light (Pl. 3, figs. 5, 6).

(c) SHORT PERIOD EXPERIMENTS, 1938–9

Each experiment was carried out with a single leaf and each epidermal strip was taken within the area previously enclosed by the porometer cup. The original purpose of the experiments was to obtain for each leaf used both porometer readings and Lloyd's strips over as wide a range of stomatal apertures as possible; the usual but not invariable procedure was therefore to obtain the widest aperture by prolonged illumination with the cup in one position in the morning, when the 'opening tendency' was greatest, and to obtain the smaller apertures during the afternoon and evening by suitable periods of darkness or light. The treatments therefore lack the regularity and symmetry that could have been assured in a series of experiments expressly designed for the present purpose, but nevertheless the results are of some interest.

The source of light was a 1000 W. tungsten filament lamp in 1938 and a 200 W. lamp in 1939, both about 1 ft. distant from the leaf and with a running-water screen interposed. Clones 5 and 3 (Heath, 1941) were used and all the experimental leaves appeared healthy and were of more than 4 in. diameter.

Experiment of 25 *July* 1938

Clone 5. Leaf petiole cut under water and attached to a water supply (Gregory, 1938) 10.40 a.m. The porometer cup was first attached at 10.50 a.m. and the leaf left in darkness for 26 min. to recover from the 'shock effect' of fixing the cup. The course of the experiment is indicated in Table 3. Photographs are presented for strips nos. 1, 3 and 6 (Pls. 1 & 8, figs. 9–18). The smaller mean aperture in no. 1 shows clearly in the low-power photograph (Pl. 1, fig. 9), but changes in average starch content are not sufficiently marked to be recognized with certainty in these photographs. Perhaps a slight decrease from no. 1 to no. 3 and a slight increase from nos. 1 and 3 to no. 6 are visible (Pl. 1, figs. 9–11). In the high-power photographs it is seen that in no. 3, after 3½ hr. illumination (1 hr. since fixing the cup) and with the aperture doubled as compared with no. 1, there is a distinct reduction in the minimum starch content but no obvious change in the maximum (Pl. 8, figs. 13, 15; 12, 14). In no. 6, after a further 2½ hr., in the light except for three very brief periods, there is virtually no change in the apertures of the stomata photographed under high power (Pl. 8, figs. 17, 18), but whereas the maximum starch content is again apparently unaltered the minimum starch shows a great increase. The 'minimum starch' stoma of no. 3 is much smaller than that of no. 6, and small stomata generally tend to have less starch, but even if the much larger 'medium starch' stoma from no. 3 (Pl. 8, fig. 16) were taken as the minimum there would still be shown an increase from no. 3 to no. 6. In this experiment, therefore, although the maximum starch content shows no change, the minimum starch content shows a decrease from 11.45 a.m. (no. 1) to 4.4 p.m. (no. 3) and an even greater increase from 4.4 p.m. (no. 3) to 6.37 p.m. (no. 6); average starch contents if anything show similar changes. The increase of stomatal starch in the late afternoon is apparently independent of stomatal movement.

Table 3. *Experiment of* 25 *July* 1938

Time	Duration (min.)	Light or darkness	Mean stomatal width (μ)	Stomatal movement (porometer)	Remarks
10.50 a.m.					Cup fixed
	26	D			
11.16 a.m.					
	9	L			
11.25 a.m.					
	20	D		Opening	
11.40 a.m.					
				Stationary	
11.45 a.m.			*4·3*		*Strip no. 1*
	24	L			
12.9 p.m.					Cup fixed
	20	D		Closing	
12.29 p.m.			3·4		Strip no. 2
	18	L			
12.47 p.m.					Cup fixed
	113	L		Opening	
2.40 p.m.					Stripping failed
	19	L			
2.59 p.m.					Cup fixed
	65	L		Opening	
4.4 p.m.			*8·3*		*Strip no. 3*
	30	L			
4.34 p.m.					Cup fixed
	10	L		Opening	
4.44 p.m.					
	5	D		Opening	
4.48 p.m.					
				Stationary	
4.49 p.m.			6·0		Strip no. 4
		L			
5.18 p.m.					Cup fixed
	17	L		Opening	
5.35 p.m.					
	4	D		Closing	
5.39 p.m.			4·8		Strip no. 5
	26	L			
6.5 p.m.					
	29	L		Opening	
6.34 p.m.					
	3	D		Opening	
6.36 p.m.					
				Stationary	
6.37 p.m.			*7·0*		*Strip no. 6*

Experiment of 13–14 *October* 1938

Clone 3. Leaf petiole cut off under water and attached to water supply at 10.50 a.m. The course of the experiment is set out in Table 4.

The low-power photographs for strips nos. 44–46 (Pl. 4, figs. 19–21) suggest that with practically no change in mean aperture there is some increase in stomatal starch during the afternoon. In the high-power photographs, with negligible change of aperture, this increase is clearly shown both in the maximum and minimum starch contents of no. 46 (Pl. 9, figs. 26, 27). The 'minimum starch' stomata of both no. 46 (Pl. 9, fig. 27) and no. 45 (Pl. 9, fig. 24) actually had rather more starch than shows in the photographs, owing to some chloroplasts lying out of the plane of focus; in no. 44 (cf. Pl. 8, fig. 23) some *small* stomata were practically starch-free. The 'maximum starch' stoma of no. 45 was not photographed but even the 'medium starch' stoma figured (Pl. 8, fig. 25) shows

Table 4. *Experiment of* 13–14 *October* 1938

Time	Duration (min.)	Light or darkness	Mean stomatal width (μ)	Stomatal movement (porometer)	Remarks
11.6 a.m.					Cup fixed
	138	L		Opening	
1.24 p.m.			8·1		*Strip no. 44*
	13	L			
1.37 p.m.					Cup fixed
	67	L		Opening	
2.44 p.m.			7·9		*Strip no. 45*
	19	L			
3.3 p.m.					Cup fixed
	59	L		Opening	
4.2 p.m.					Stripping failed
	10	L			
4.12 p.m.					Cup fixed
	48	L		Opening	
5.0 p.m.					Stripping failed
	8	L			
5.8 p.m.					Cup fixed
	26	L		Opening	
5.34 p.m.			6·7		*Strip no. 46*
	201	L and D			Cup fixed and strips taken four times
8.55 p.m.					Cup fixed
	5	L			
9.0 p.m.					
	780	Natural daylight and darkness			
10.0 a.m.					
	43	L		Closing	
10.43 a.m.					
	23	D		Stationary	
11.6 a.m.			2·3		*Strip no. 50* (leaf wilted)

as much starch as the maximum in no. 44 (Pl. 8, fig. 22). In this experiment again, therefore, an increase of stomatal starch during the afternoon, independent of light and stomatal aperture, is found.

Strip no. 50 was taken when the detached leaf had wilted and all the stomata were nearly or quite closed. The maximum and minimum starch contents of practically closed stomata are shown in the high-power photographs (Pl. 9, figs. 28, 29). Since partly open stomata in this strip showed a similar range of starch contents the low-power photograph is not reproduced. The very heavy starch contents due to wilting are clearly shown.

Experiment of 24–25 *October* 1939

Clone 5. Leaf attached to plant, left darkened overnight with a porometer cup in position. The subsequent course of the experiment is shown in Table 5.

In the low-power photographs little difference in mean starch content is apparent between nos. 81 and 84 (Pl. 5, figs. 30, 31); in no. 86 (Pl. 5, fig. 32), however, in spite of the greatly reduced mean aperture due to overnight darkness the average starch content is apparently less than in either no. 81 or no. 84, for the maximum starch is about the same but the minimum is greatly reduced. The average starch content of no. 89 cannot be judged from the low-power photograph (Pl. 6, fig. 33). The high-power photographs show little difference in maximum starch content between any of the strips (Pl. 9, figs. 34, 36, 38), but with such very abundant starch it is difficult to judge. Minimum

Table 5. *Experiment of* 24–25 *October* 1939

Time	Duration (min.)	Light or darkness	Mean stomatal width (μ)	Stomatal movement (porometer)	Remarks
11.32 a.m.		D	3·1	Stationary	Strip no. 80
	85	L			
12.57 p.m.					Cup fixed
	91	L		Opening	
2.28 p.m.			*7·6*	Stationary	*Strip no. 81*
	17	L			
2.45 p.m.					Cup fixed
	35	L		Opening	
3.20 p.m.			4·8		Strip no. 82
	25	L			
3.45 p.m.					Cup fixed
	30	L		Opening	
4.15 p.m.			5·1		Strip no. 83
	28	L			
4.43 p.m.					Cup fixed
	17	L		Almost stationary	
5.0 p.m.					
	20	D		Almost stationary	
5.20 p.m.					
	1	L		Stationary	
5.21 p.m.			*5·4*		*Strip no. 84*
	26	L			
5.47 p.m.					Cup fixed
	28	D		Closing	
6.15 p.m.			4·7	Stationary	Strip no. 85
	25	L			
6.40 p.m.					Cup fixed
	905	D			
9.45 a.m.					
	24	D		Closing	
10.9 a.m.			*3·4*		*Strip no. 86*
	26	L			
10.35 a.m.					Cup fixed
	213	L		Opening	
2.8 p.m.			8·0	Stationary	Strip no. 87
	36	L			
2.44 p.m.					Cup fixed
	92	L			
4.16 p.m.			6·5 approx.		Strip no. 88
	20	L			
4.36 p.m.					Cup fixed
	17	L		Opening	
4.53 p.m.			*7·4*		*Strip no. 89*

starch shows some interesting changes, however, for although there is little difference in quantity between nos. 81 and 84 (Pl. 9, figs. 35, 37), no. 86 shows a striking reduction (Pl. 9, fig. 39) and even the very large closed stoma with 'medium starch' figured (Pl. 9, fig. 40) shows somewhat less than the minimum starch of no. 84 (Pl. 9, fig. 37). The minimum starch of no. 89 (Pl. 10, fig. 42) shows in spite of stomatal opening a distinct increase as compared with no. 86 (Pl. 9, fig. 39). In this experiment, therefore, stomatal closure in darkness is accompanied, in the morning, by a decreased minimal starch content; stomatal opening in light, in the afternoon, is accompanied by an increase in minimal starch. Any effect of light on stomatal starch is thus apparently overridden by the diurnal rhythm.

Experiment of 16–17 *November* 1939

Clone 3. Porometer cups fixed, to attached leaf, overnight and readings taken from 9.40 a.m. 16 November 1939. Details are given in Table 6.

Photographs are presented for strips nos. 139, 143, 144, 145 and 146. In the low-

Table 6. *Experiment of* 16–17 *November* 1939

Time	Duration (min.)	Light or darkness	Mean stomatal width (μ)	Stomatal movement (porometer)	Remarks
2.2 p.m.					
	184	D		Closing	
5.6 p.m.			*3·8*		*Strips nos. 139 and 140*
	74	L			
6.20 p.m.					Cup fixed
	940	D			
10.0 a.m.					
	25	D		Closing	
10.25 a.m.			6·0		Strip no. 141
	28	L			
10.53 a.m.					Cup fixed
	190	L		Opening Stationary	
2.3 p.m.			8·8		Strip no. 142
	20	L			
2.23 p.m.					Cup fixed
	53	L		Opening	
3.16 p.m.			*7·2*		*Strip no. 143*
	29	L			
3.45 p.m.					Cup fixed
	43	L		Opening	
4.28 p.m.			*6·8*		*Strip no. 144*
	20	L			
4.48 p.m.					Cup fixed
	41	L		Opening	
5.29 p.m.			*6·8*		*Strip no. 145*
	26	L			
5.55 p.m.					Cup fixed
	13	L		Opening	
6.8 p.m.					
	6	D		Opening	
6.14 p.m.					Stripping failed
	9	L			
6.23 p.m.					Cup fixed
	28	L		Opening	
6.51 p.m.			*4·9*		*Strip no. 146*

power photographs, the much wider mean apertures of no. 144 (Pl. 7, fig. 45) and of no. 145 (Pl. 7, fig. 46) than of no. 139 (Pl. 6, fig. 43) are seen to be accompanied by, if anything, higher average starch contents; this is in spite of the fact that the strips were taken at *almost* the same time of day and that for no. 145 the leaf had been illuminated for 7 hr. while for no. 139 it had been in darkness for 3 hr. There is no obvious change to be seen between no. 143 (Pl. 6, fig. 44), no. 144 (Pl. 7, fig. 45) and no. 145 (Pl. 7, fig. 46), either in mean aperture or average starch content; no. 146 (Pl. 7, fig. 47) shows no obvious change in average starch although the apertures are much reduced. In the high-power photographs it appears that the minimum starch content in no. 145 (Pl. 10, fig. 55) is higher than in no. 143 (Pl. 10, fig. 51) earlier in the afternoon with the same aperture, and if anything higher than in no. 139 (Pl. 10, fig. 49) in which the stomata were nearly closed in the dark at the same time on the previous day. In no. 146, the minimum starch content (Pl. 10, fig. 57) shows little change. As far as maximum starch content is concerned, no. 145 (Pl. 10, fig. 54) and no. 144 (Pl. 10, fig. 52) appear to have somewhat less than no. 139 (Pl. 10, fig. 48), and this might be attributed to a light effect though the apparent converse differences in minimum and average starch content must be remembered. Taking the evidence as a whole there is little indication of a difference in starch due to light. The maximum starch content shows a progressive increase from no. 143 (Pl. 10, fig. 50) to no. 144 (Pl. 10, fig. 52), no. 145 (Pl. 10, fig. 54) and thence to no. 146 (Pl. 10, fig. 56) with little difference in width of aperture between the first three. This is evidence of the usual increase in starch in the late afternoon independent of aperture, or possibly of the effect of shorter periods of enclosure under the cup causing less reduction in starch (see p. 207).

DISCUSSION

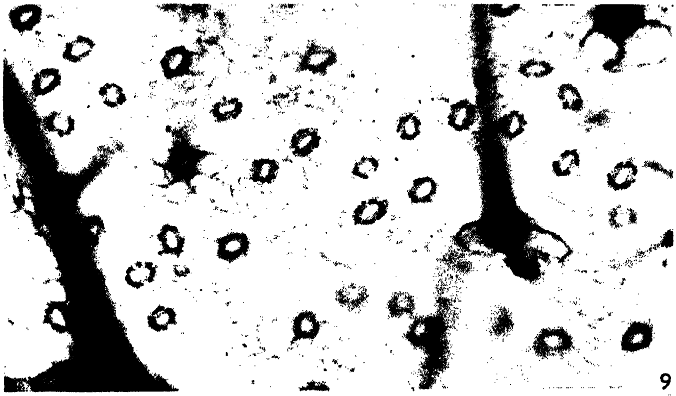
A brief historical review of the literature relevant to the role of starch in the light response of stomata has been presented in the Introduction, but it seems desirable to discuss in rather more detail the results obtained by some of the previous workers before considering the implications of the data collected in the present investigation. In particular, the results obtained by Lloyd (1908) with light and other conditions artificially varied require further scrutiny. His experiment, in which the stomata remained motile when all guard-cell starch had disappeared as a result of exposure to blue light, has already been discussed (p. 187). Unfortunately, as already mentioned, there is the possibility that this apparent light response in the complete absence of stomatal starch might in fact have been due to a diurnal rhythm, since the blue light was given during the normal hours of daylight. In other experiments in which *Verbena ciliata* cuttings were placed in darkness in the early morning, stomatal movement showed little correlation with starch content. Thus in his exp. no. 144 (p. 115) considerable stomatal closure occurred between 8 a.m., when the cutting was placed in the dark, and 11 a.m., but at the latter time the starch in the guard cells was 'little or none'. By 1 p.m. starch had appeared in the guard cells but no further change had occurred in stomatal aperture. By 8 a.m. the next day the guard-cell starch had increased to 'plenty' but the stomata showed a slight *increase* of aperture. In his exp. no. 154 (p. 116) a cutting placed in the dark at 8.55 a.m. and a control cutting kept in the light both showed considerable stomatal closure by 10.30 a.m. with 'no increase observable' in starch contents. In his exp. no. 155 (p. 116) cuttings were kept in the dark for 4 days. At 8.35 a.m., at the beginning of the experiment, the stomata were open and starch was absent; at 8.50 a.m. on the second day the stomata were nearly all closed and starch was 'abundant in large granules'; at 8 a.m. on the third day even more of the stomata were closed but starch was present 'only in an occasional stoma'; finally, at 7 a.m. on the fourth day with some stomatal opening the record for starch was 'varies from little to abundant'. These results are more suggestive of random fluctuation than a causal connexion between starch content and aperture. In his exp. no. 160 (p. 118), one cutting was placed in the dark at 6.50 a.m. and another kept in the light. Starch was then present as 'fine granules'. By 11.30 a.m. the darkened cutting showed little change of aperture and 'no visible change' of starch, but the control showed a very great increase in stomatal width, also with 'no obvious change' in starch. In an experiment with CO_2-free air (no. 162, p. 123) Lloyd found that between 9.30 a.m. and 5.45 p.m. the starch in the stomata changed from 'minute granules' to 'abundant in the guard cells', while the pore width was 4–8μ on each occasion. Even the data for normal diurnal changes in stomatal aperture and starch content presented by Lloyd do not show an invariable correlation. Thus his table 48 (p. 98) shows apparently good correlation between starch changes and stomatal movement for 7 July, but for 8, 12 and 17 July the records of one or the other variable are too incomplete to be convincing. On 10 July practically all the stomatal opening, from 3 to 5.30 a.m., occurred while starch changed from 'present' to 'abundant'; the disappearance of starch by 8.20 a.m. and its increase again by 9 a.m. were unaccompanied by appreciable further stomatal movement.

Sayre's (1926) data for stomatal apertures and starch contents in *Rumex patientia* do not always show correlated changes. As mentioned in the Introduction, the midday closure which occurred was independent of both stomatal starch and osmotic pressure of the guard cells. He attributed this closure to turgor changes in the surrounding cells, but such changes are usually held responsible for the stomatal *opening* which occurs in the early stages of wilting (Laidlaw & Knight, 1916), and which was also found by Sayre when leaves were detached from the plant. With permanent wilting considerable changes of osmotic pressure and starch content were found. In his Table 8, however, in which no midday closure is shown, from 1 to 3 p.m. the stomatal width decreased from 17 to 11 μ with no change in guard-cell starch. The following day, from 9 to 11 a.m. the width increased from 6 to 9 μ, again without change of starch content.

Sayre's major contribution lay in the discovery of the effects of pH changes upon guard-cell movements. He found that guard cells of open stomata showed a higher pH with bromphenol blue than those of closed stomata and this led him to try the effects of buffer solutions of various pH on stomatal opening in detached epidermis. He tried various buffer mixtures, of which the most satisfactory and least toxic was prepared by dialysis of juice expressed from the leaves. This produced a clear liquid of pH 3·8–3·9, which was then adjusted to the desired pH with HCl or NaOH. Using this and other buffer solutions he found maximal stomatal opening with a pH of 4·2–4·4 with closure on either side of this range. Such opening was accompanied by an increase in osmotic pressure and sugar content of the guard cells and a decrease in their starch content. With closure the converse changes occurred. Sayre next tried the effects of ammonia and acetic acid vapours on the stomata of intact leaves. Ammonia vapour caused stomatal opening in darkness to 50% of the maximum; acetic acid vapour caused closure of open stomata in the light. On the basis of these results Sayre put forward the hypothesis that has already been given in brief (p. 175).

Scarth (1929, 1932), who continued this work using *Tradescantia zebrina*, confirmed Sayre's findings for the effects of ammonia and acetic acid vapours, although stomatal opening occurred with the former only when the epidermal cells had changed from their normal purple colour to green and the green guard cells and subsidiary cells to yellow, i.e. at a pH of about 7·4 (see Scarth, 1932, p. 491). He also found stomatal movements on immersion of epidermal strips or leaf sections in dilute solutions of ammonia and acetic acid (Scarth, 1929). From pH 5·0 to 11·0 the stomata passed from closure to wide opening; practically all the movement occurred between pH 7·5 and 11·0, however, and thus lay outside the natural range (pH 4·5–7·0) claimed by Scarth to have been found in stomata on intact leaves in dark and light respectively. Values on the alkaline side of pH 7·0 in any case seem very improbable and the approximate natural pH ranges found by Small & Maxwell (1939) and Small *et al.* (1942) in guard cells on intact leaves vary from pH 4·0–4·4 to 4·0–5·6 for different species.

As already noted, by immersing detached epidermis in a variety of buffer mixtures Small *et al.* (*loc. cit.*) obtained violent fluctuations in both stomatal aperture and starch content with pH, and the positions of maxima and minima varied markedly with the type of buffer mixture used. It was thus possible, in general, to find some buffer mixture in which stomatal movement occurred in a pH range similar to that found in the stomata



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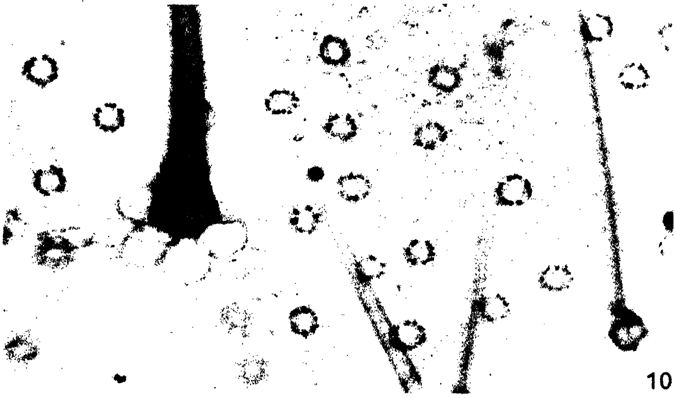

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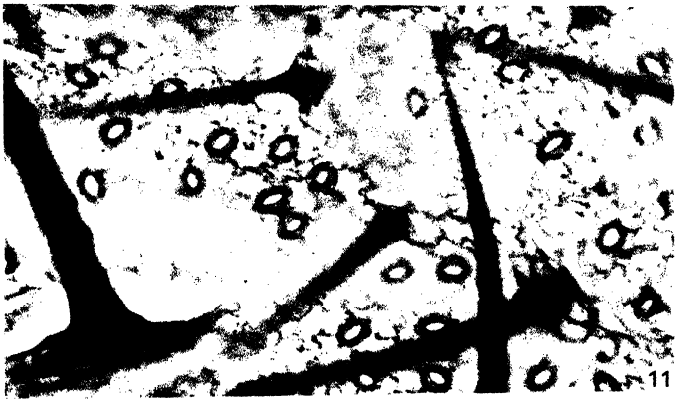

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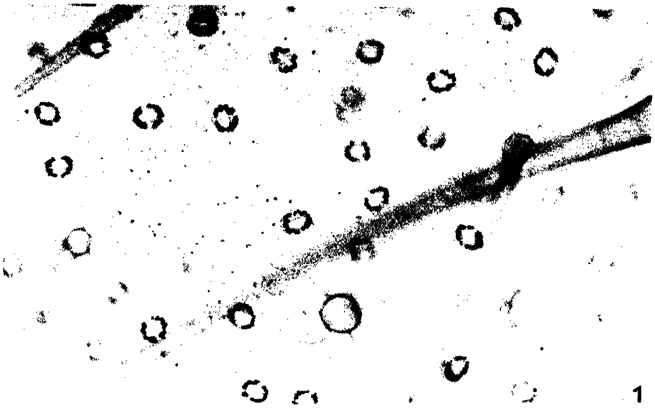
HEATH—STUDIES IN STOMATAL BEHAVIOUR



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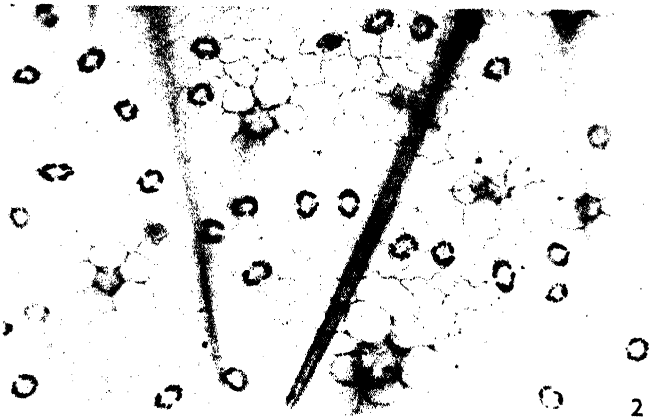

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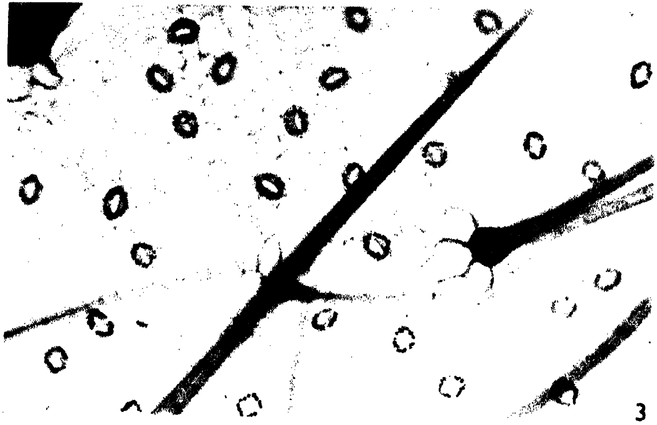

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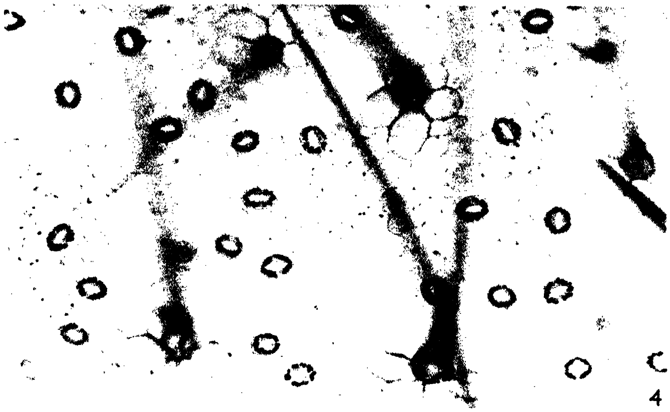

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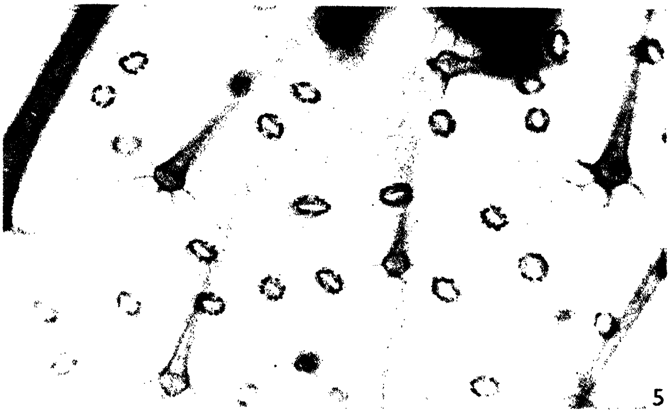

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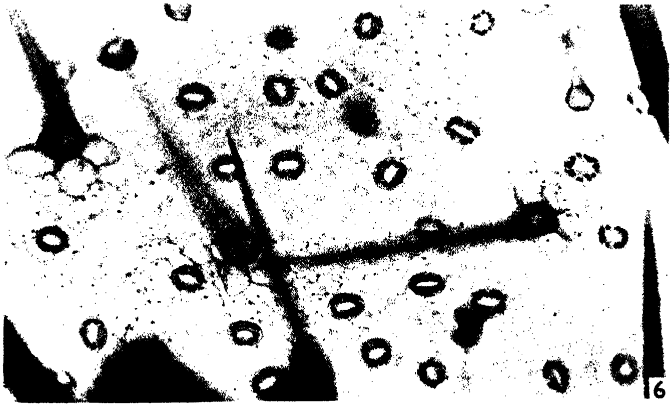

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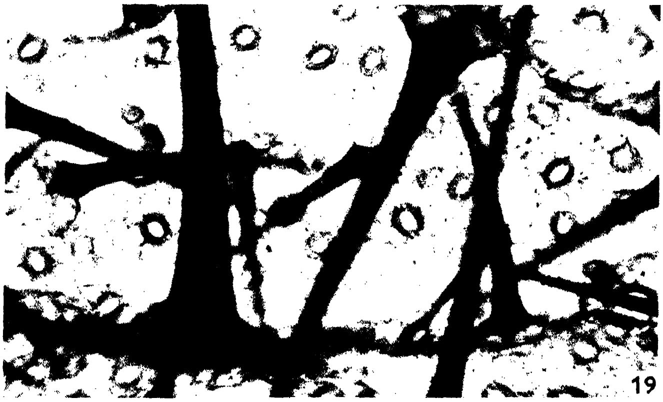

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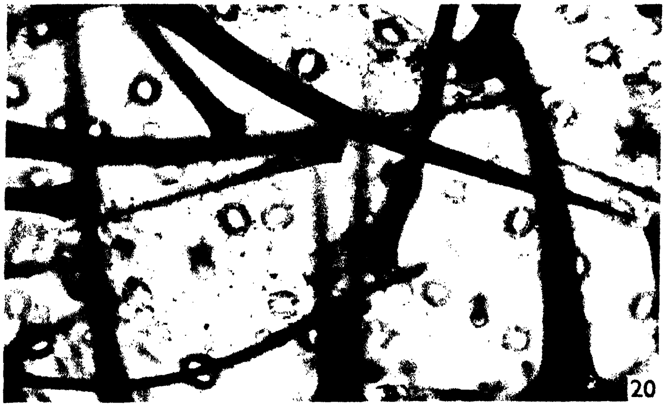

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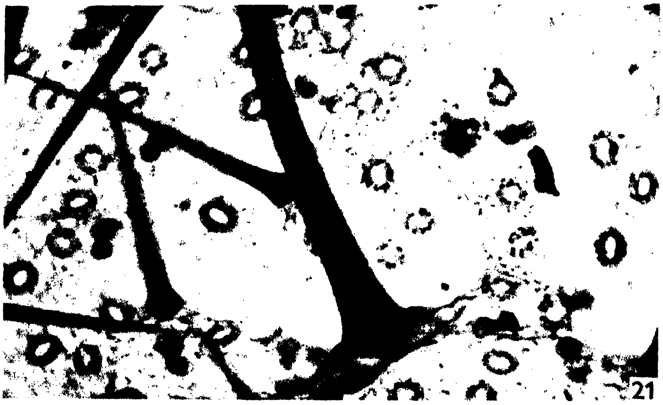

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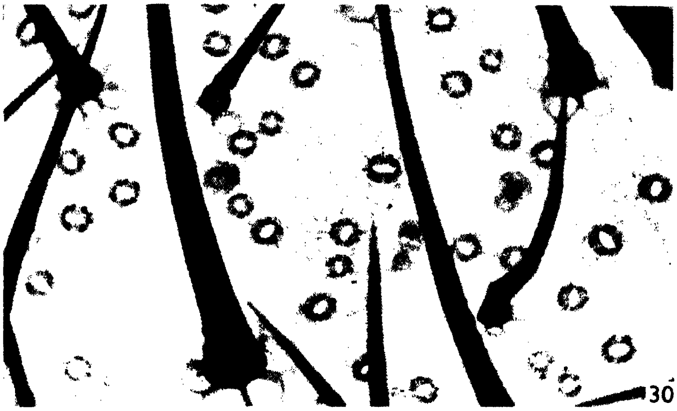

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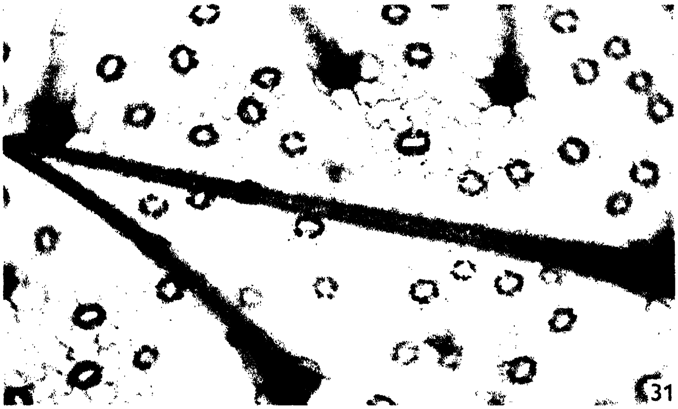

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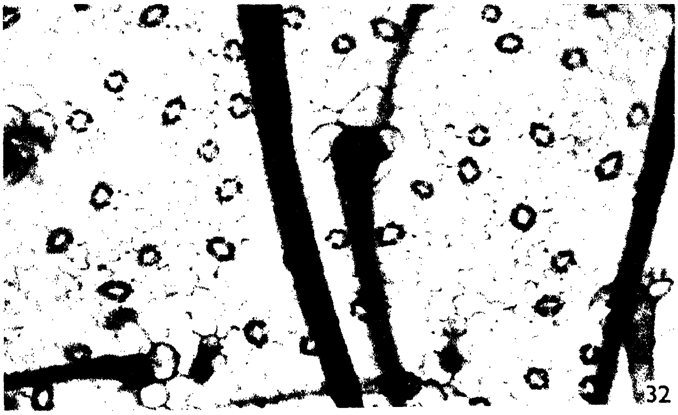

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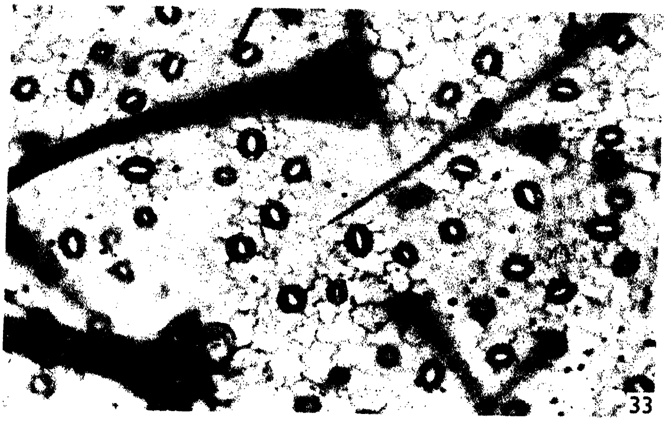

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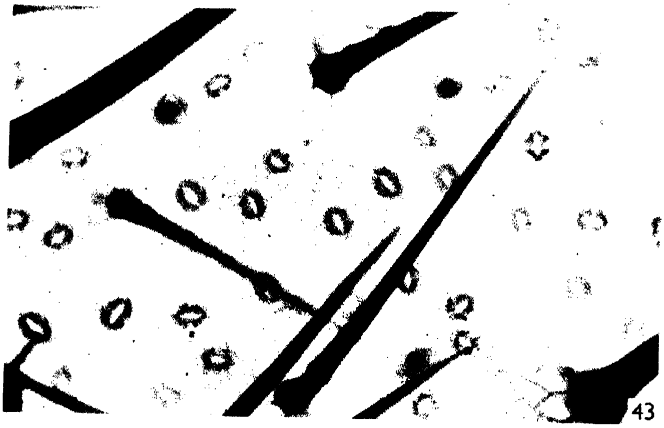

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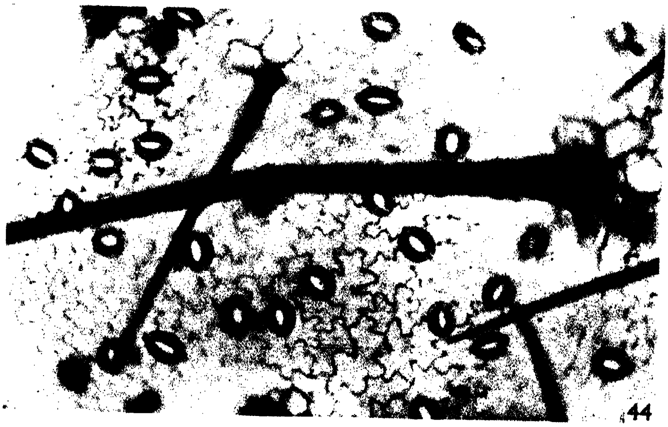

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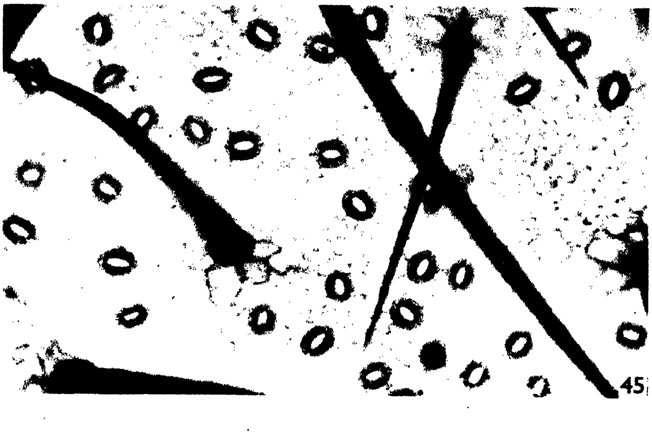
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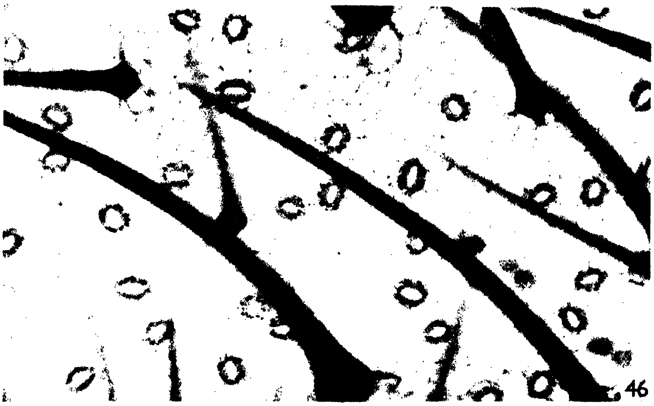
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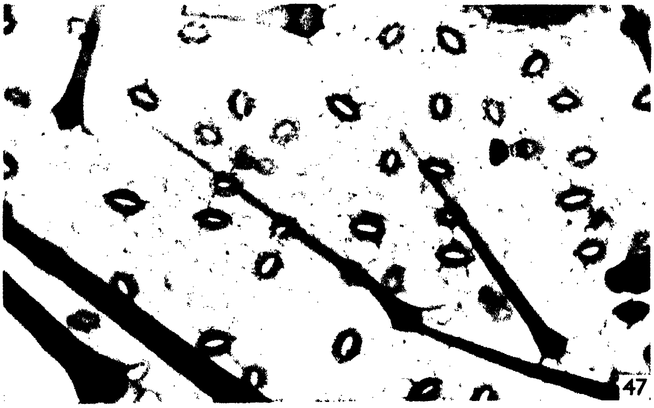
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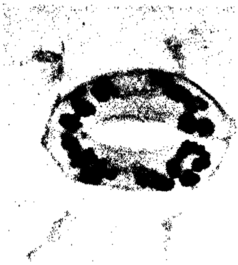

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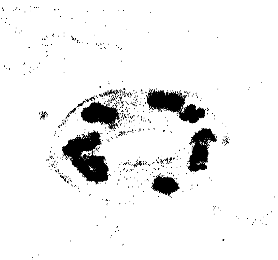

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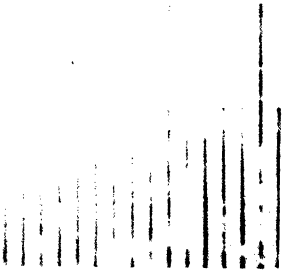

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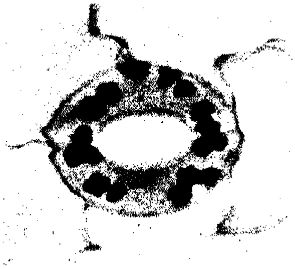

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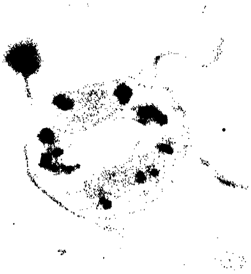

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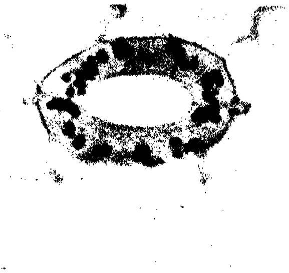

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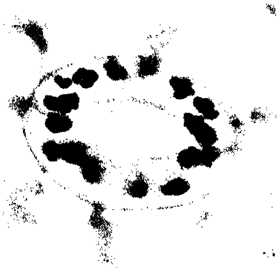

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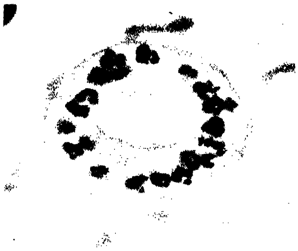

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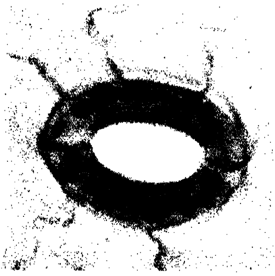

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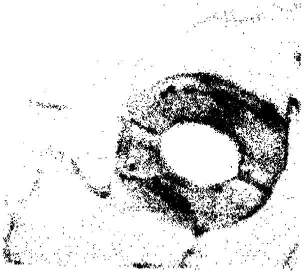

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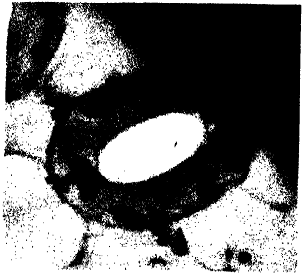

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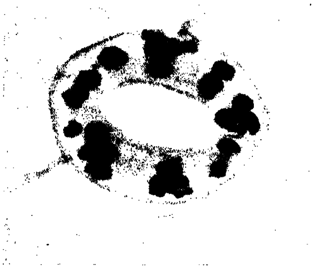
HEATH—STUDIES IN STOMATAL BEHAVIOUR



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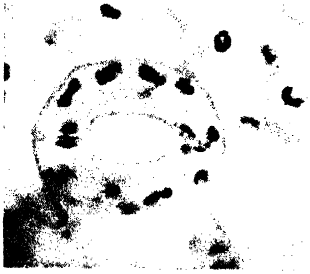

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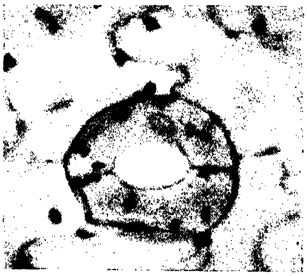

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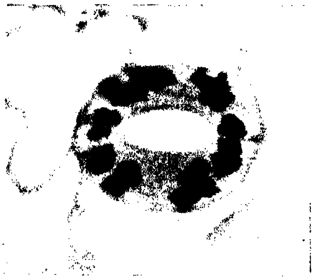

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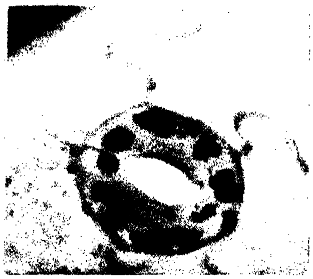

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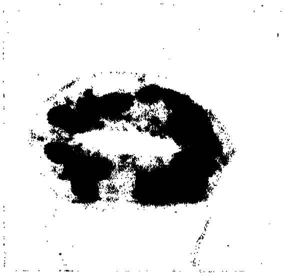

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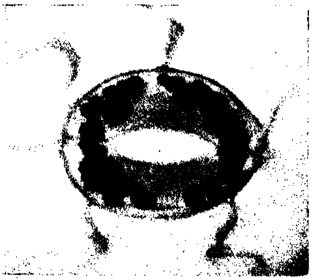

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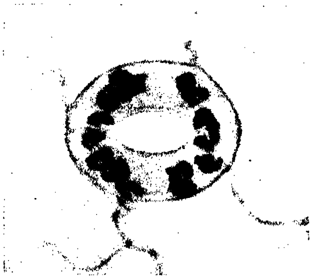

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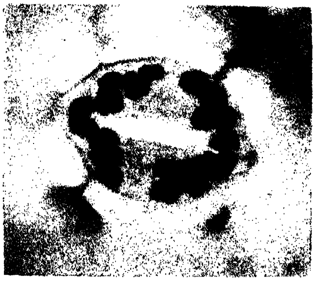

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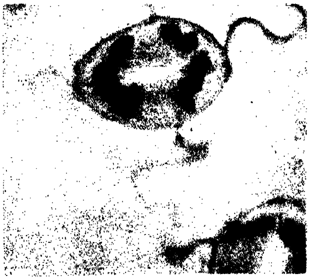

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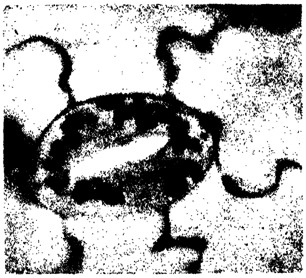

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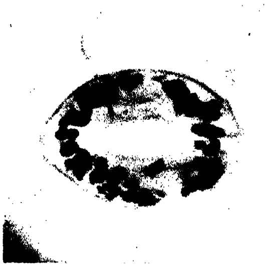

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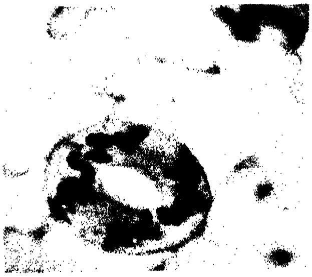

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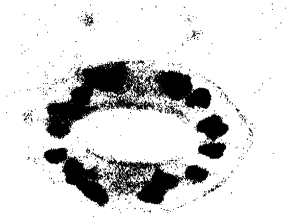

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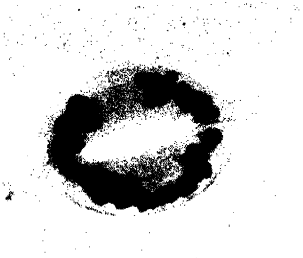

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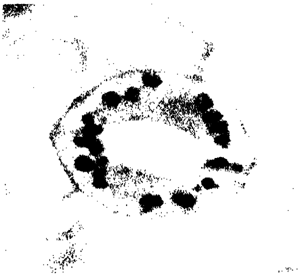

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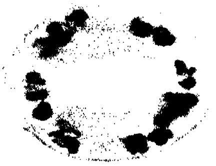

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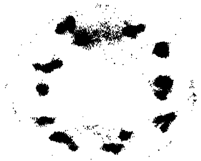

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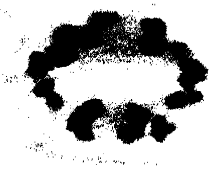

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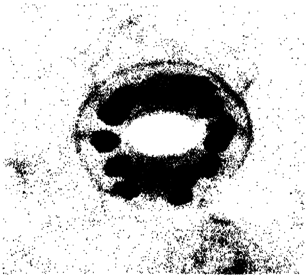

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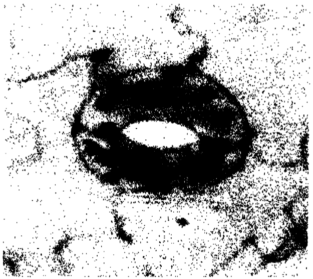

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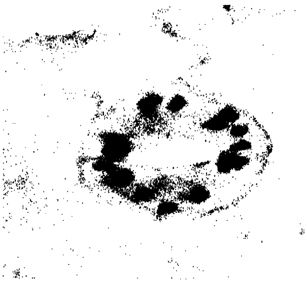

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on intact leaves of the same species, but in view of the complex effects of salts on stomata in detached epidermis found by Arends (1926) and others, this is perhaps not surprising.

The work of Hanes (1940) on phosphorylase lends new importance to the question of control of starch hydrolysis and synthesis in the guard cells by pH changes. He found that with potato phosphorylase the equilibrium between starch plus free phosphate and glucose-1-phosphate depended upon the pH: with a pH change from 5·4 to 6·4 the ratio of free phosphate to ester phosphate changed from 9/1 to 5/1. This is within the natural pH range found by Scarth in stomata on intact leaves, although outside the range recorded by Small *et al.* for most species.

It should again be emphasized, however, that neither in the work of Sayre, of Scarth, nor of Small *et al.*, are any direct experiments upon the effect of *light* on stomatal starch described. If the light response of the stomata is in fact due to a pH change there is little evidence that it acts through the hydrolysis of starch. Furthermore, the method by which light produces a pH change in the guard cells is still quite uncertain. It has recently been found that very small changes in the carbon-dioxide concentration of the air are sufficient to cause considerable stomatal movement (Heath, 1948), and although it might be argued that such small changes would affect the pH of the guard cells this seems unlikely to occur to any appreciable extent in what must be a buffered system.

The claims of Semmens (1947) that polarized light causes opening of stomata and hydrolysis of stomatal starch have been mentioned in the Introduction. The results from two of her experiments with entire leaves (I and VIII) may be easily explained as due to carbon-dioxide depletion where the leaf was covered by the Nicol prism,* for the conditions there must have been very similar to those inside a closed porometer cup (cf. Heath & Williams, 1948; Heath, 1948). Her exp. V in which two *Tropaeolum* plants were enclosed in chambers, one lighted by partly polarized and the other by ordinary light, is not open to this criticism. The difference in stomatal starch between a pair of leaves, one from each plant, as indicated by the published photograph was not striking, however, and as has been found in the present work leaf to leaf variations in this respect are often considerable (cf. Exp. 9, p. 195). Her claims for the effects of polarized light on the stomata on detached epidermis, if substantiated, raise theoretical questions which cannot be dealt with here. Her findings that such stomata do not respond to ordinary light is in agreement with the results so far obtained by the present author.

In connexion with the apparent lack of light response of stomata in detached epidermal strips, it seems worth while to place on record the observation that in onion and *Pelargonium* many or most of the epidermal cells are killed by the act of stripping off the epidermis, as is indicated by the fact that they do not take up neutral red in the vacuoles. It has been found that onion stomata, on strips of epidermis taken into water, are invariably wide open when flanked by two dead epidermal cells and almost invariably closed when flanked by two living epidermal cells. This state of affairs continues irrespective of lighting. *Pelargonium* stomata, under similar conditions, appear little if at all affected by whether the adjacent epidermal cells are living or dead; they show a tendency to open very slowly in water, over many hours, irrespective of the lighting conditions.

The very great variability both in the starch contents and apertures of stomata, even within a small area of epidermis, are well known and are clearly demonstrated in the photographs presented. This renders the selection of an 'average stoma' extremely difficult and for this reason stomata showing maximal and minimal starch contents were selected for the high-power photomicrographs. The selection of the widest open stomata from the light and of those relatively closed from the dark made it probable that stomata which had responded markedly to the light or dark stimulus were photographed. (Damaged or dead stomata, which are usually closed, were of course avoided.) As already mentioned, for previous workers the difficulties of estimation of the relation between stomatal aperture and starch content were increased by the impossibility of observing both aperture and starch simultaneously in the same stoma, and subjective errors must have been considerable. Although such errors are reduced by the use of the reagent described (p. 194) and by comparison of photomicrographs, the difficulties of taking representative samples and estimating mean values remain. Since no quantitative measure of stomatal starch has been devised or indeed seems possible, and since the great variability mentioned so baffles the judgement that only the crudest and most approximate allocation of marks can be achieved, the quantitative comparison of treatment effects with the random variation has not been attempted in this or, the writer believes, in any other published work; nor has even a qualitative survey of the variation in stomatal starch between leaves and within leaves under constant conditions been carried out. Many of the changes in stomatal starch attributed to external factors or time of day in this and other investigations may in fact, therefore, be purely random fluctuations. Nevertheless, the cumulative effect of the work of Lloyd, Rosing, Hagen, Loftfield and Sayre, considered in the Introduction, is to leave virtually no doubt that there is normally a diurnal rhythm in stomatal starch content and some evidence of such a rhythm has been found in some of the present experiments with *Pelargonium*. In the case of stomatal aperture a diurnal rhythm has been found in *Pelargonium* even under constant conditions (Gregory & Pearse, 1937). In the experiments described in section *b*, however, any effect of such rhythms was expressly excluded by taking epidermal strips from leaves in the light and dark at the same time of day. It was suggested by the results of Exp. 9 that comparisons of stomatal starch between successive days for the same leaf were preferable to those between different leaves on the same day, since leaf-to-leaf variation could greatly exceed treatment effects due to light and darkness. About 10 a.m. was selected as the time for observations, since stomatal starch was then expected to be minimal and therefore differences should be easiest to estimate. In all the experiments of this section, differences in the environments of the plants in light and darkness must have existed also in respect of temperature, atmospheric humidity and carbon-dioxide concentration. In the three earlier experiments, however (Exps. 8, 9 and 12), the leaves showed appreciably wider stomatal apertures when illuminated than in the dark, and there seems no reason to suppose that this was not a genuine light response. Nevertheless, there was no evidence that the opening in the light was accompanied by a reduction of starch in the guard cells —if anything the reverse occurred. In Exp. 30 the experimental leaf of one plant (B) showed little difference in stomatal aperture after any of the 24 hr. periods either with light or in darkness; the other (A) showed smaller apertures after the first period with light than after either of the adjacent 24 hr. periods of darkness, but wider apertures after the last period of illumination. This last may be reasonably attributed to light, and the lack of opening in the first 24 hr. with light was perhaps an after-effect of wilting. The lack of response in plant B is unexplained, but individual *Pelargonium* leaves are sometimes found to be very unresponsive. The stomatal starch contents in this experiment again gave no evidence of a reduction due to light.

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* I have since been informed verbally by Miss Semmens that the Nicol prism was not in actual contact with the leaf. I was misled by the statements made in her paper (Semmens, 1947) on pp. 270–1 (exp. I) and on p. 275 (exp. VIII).

Each experiment of section *c* was carried out with a single leaf and light effects were confounded with time of day to a great extent, but selected comparisons provide data which indicate that, if there is a light effect upon guard-cell starch of the kind usually postulated, it is very much overruled by a diurnal rhythm. In one experiment (16–17 November 1939) moreover, the comparison was obtained between stomata relatively closed after prolonged darkness at 5.6 p.m. on one day and those much wider open in the light at 5.29 p.m. the following day. The light effect on aperture was here unaccompanied by any appreciable difference in stomatal starch—if anything the *average* starch content was higher in the light.

A comparison of the photomicrographs for the experiments of section *c* with those of section *b* shows clearly the much wider apertures due to illumination of stomata enclosed within a porometer cup than of those freely exposed to the air (Heath & Williams, 1948). The former apertures are in fact much wider than are likely to be found in a natural state, except perhaps under conditions of crowded vegetation and completely still air when the CO_2 depletion would be maximal (Heath, 1948). This does not, however, seem to necessitate discarding the data of section *c* since there appears no reason to regard the opening of enclosed stomata as other than a normal light response. Enclosure of the stomata is thus in effect a convenient way of obtaining maximal response.

If the duration of attachment of the cup (Tables 3–6) is compared with the changes noted in stomatal starch, it is seen that in practically every case lower starch content is correlated with longer enclosure in the cup, though this is to a large extent confounded with time of day, i.e. it happened that longer enclosure generally occurred earlier in the day for the reason already noted. Thus such reductions in starch content as were observed might be thought due to prolonged enclosure in the cup rather than to a diurnal rhythm. If so, however, it would appear that such enclosure cannot affect the stomatal starch by means of the reduced CO_2 content, as is the case for the effect on stomatal aperture (Heath, 1948), for strip no. 86 (25 October 1939) and strip no. 139 (16 November 1939) both show a reduced amount of starch after very prolonged enclosure in *darkness* when the CO_2 content should have been high; possibly a humidity effect is involved.

When earlier work is also taken into account, however, it seems most probable that a real diurnal rhythm of starch content is concerned, and it seems not unlikely that such changes in the starch $\rightleftarrows$ sugar balance may provide the motive force for the diurnal rhythm in stomatal movement, both as expressed under constant conditions (Maskell, 1928; Gregory & Pearse, 1937) and as shown by the strong 'opening and closing tendencies' of stomata in the morning and evening respectively (Darwin, 1898). Unfortunately, none of the 1938–9 Lloyd's strips available was taken before and after a *short* period of illumination in the evening, when in the absence of a light effect on starch opening might be expected to be accompanied by increased starch, or before and after a *short* period of darkness in the morning, when closure and a decrease of starch might be expected. It is proposed to carry out such experiments in the future under more rigidly controlled conditions.

It is a truism that a universal negative can never be proved, and the possibility remains that in those species which form starch in the guard cells (the great majority), hydrolysis and condensation of starch may be concerned to some extent in the light-dark response of the stomata. All that can be said is that the evidence for this does not appear at present to be at all convincing, that factors other than light have much bigger effects than light itself upon the starch content of the guard cells and that light responses of the stomata may occur apparently independently of changes in stomatal starch contents.

SUMMARY

1. A review of literature on the possible rôle of starch in the light-operated mechanism of stomatal movement is presented and it is concluded that as far as the response to light is concerned the evidence for a starch $\rightleftharpoons$ sugar hypothesis is not convincing. There appears, however, little doubt that a diurnal rhythm occurs in the starch content of the guard cells. This rhythm has not been shown to be due to light and the periodic changes in hydrolysis and condensation of starch may perhaps provide the motive force for the autonomous diurnal fluctuations in stomatal aperture which have been observed in some species even under constant illumination.

2. A new reagent is described which enables the starch grains in guard cells on strips of epidermis taken by Lloyd's method to be stained without affecting the stomatal apertures as seen in such strips. Since no quantitative measure of stomatal starch seems possible, photomicrographs of preparations made with this reagent are presented to show the starch contents and apertures of stomata from the experiments described.

3. Experiments designed to eliminate any effects of diurnal rhythms, either in starch content or stomatal aperture, have failed to show any consistent differences between the starch contents of *Pelargonium* stomata with relatively wide and relatively narrow apertures under conditions of light and darkness respectively.

4. In other experiments with *Pelargonium*, increase in stomatal starch has been found in the late afternoon without appreciable change of aperture of illuminated stomata, and even decrease of starch overnight accompanied by decreased aperture of the stomata in darkness. Since no statistical tests of significance could be applied to these results, the possibility remains that they may have been caused by random variation, but at least they afford no evidence that the light-dark response of the stomata is operated by hydrolysis and condensation of starch.

REFERENCES

Alexandrov, W. G. (1925). Beiträge zur Kenntnis der Assimilations-stätigkeit der grünen Plastiden. *Ber. dtsch. bot. Ges.* **43**, 325–32.

Angelescu, E. & Mirescu, J. (1928). *J. chim. phys.* **25**, 327.

Arends, J. (1926). Über den Einfluss chemischer Agenzien auf Stärkegehalt und osmotischen Wert der Spaltöffnungs-schliesszellen. *Planta*, **1**, 84–115.

Ashby, E. (1931). Comparison of two methods of measuring stomatal aperture. *Plant Physiol.* **6**, 715–19.

Darwin, F. (1898). Observations on stomata. *Philos. Trans.* B, **190**, 531–621.

Gregory, F. G. (1938). A convenient method for attaching potometers and an example of its use in measuring the uptake of water by leaves during recovery from wilting. *Ann. Bot., Lond.*, N.S. **2**, 253–5.

Gregory, F. G. & Pearse, H. L. (1934). The resistance porometer and its application to the study of stomatal movement. *Proc. Roy. Soc.* B, **114**, 477–93.

Gregory, F. G. & Pearse, H. L. (1937). The effect on the behaviour of stomata of alternating periods of light and darkness of short duration. *Ann. Bot., Lond.*, N.S. **1**, 3–10.

Hagen, F. (1918). Zur Physiologie des Spaltöffnungsapparates. *Beitr. Allg. Bot.* **1**, 261–91.

Hanes, C. S. (1940). Enzymic synthesis of starch from glucose-1-phosphate. *Nature, Lond.*, **145**, 348.

Heath, O. V. S. (1938). An experimental investigation of the mechanism of stomatal movement, with some preliminary observations upon the response of the guard cells to 'shock'. *New Phytol.* **37**, 385–95.

Heath, O. V. S. (1941). Experimental studies of the relation between carbon assimilation and stomatal movement. II. The use of the resistance porometer in estimating stomatal aperture and diffusive resistance. Part 1. A critical study of the resistance porometer. With an appendix by H. L. Penman. *Ann. Bot., Lond.*, N.S. **5**, 455–500.

Heath, O. V. S. (1947). Role of starch in light-induced stomatal movement, and a new reagent for staining stomatal starch. *Nature, Lond.*, **159**, 647.

Heath, O. V. S. (1948). Studies in stomatal action. Control of stomatal movement by a reduction in the normal carbon dioxide content of the air. *Nature, Lond.*, **161**, 178.

Heath, O. V. S. & Williams, W. T. (1948). Studies in stomatal action. Adequacy of the porometer in the investigation of stomatal aperture. *Nature, Lond.*, **161**, 178.

Iljin, W. S. (1914). Die Regulierung der Spaltöffnungen im Zusammenhang mit der Veränderung des osmitischen Druckes. *Beih. Bot. Zbl.* **32**, 15–35.

Kisser, J. (1930). Die Verwendung von Jod-Phenol zum histochemischen Nachweis kleinster Stärkemengen. *Mikrochem. Emich. Festschrift*, pp. 175–9.

Kohl, F. G. (1895). Über Assimilations-energie und Spaltöffnungsmechanik. *Bot. Zbl.* **64**.

Laidlaw, C. G. P. & Knight, R. C. (1916). A description of a recording porometer and a note on stomatal behaviour during wilting. *Ann. Bot., Lond.*, **30**, 47–56.

Lloyd, F. E. (1908). The physiology of stomata. *Publ. Carneg. Instn*, **82**, 1–142.

Loftfield, J. V. G. (1921). The behaviour of stomata. *Publ. Carneg. Instn*, **314**, 1–104.

Maskell, E. J. (1928). Experimental researches on vegetable assimilation and respiration. XVIII. The relation between stomatal opening and assimilation. A critical study of assimilation rates and porometer rates in leaves of cherry laurel. *Proc. Roy. Soc.* B, **102**, 488–533.

Maximov, N. A. (1938). *Textbook of Plant Physiology*, 2nd ed. New York.

von Mohl, H. (1856). Welche Ursachen bewirken die Erweiterung und Verengung der Spaltöffnungen? *Bot. Zbl.* **14**, 697–704, 713–20.

Newton, R. G. (1936). The investigation of the relation between rates of assimilation and stomatal aperture in leaves. Ph.D. Thesis, Univ. of London.

Nutman, F. J. (1937). Studies in the physiology of *Coffea arabica*. II. Stomatal movements in relation to photosynthesis under natural conditions. *Ann. Bot., Lond.*, N.S. **1**, 681–94.

Parkin, J. (1899). Contributions to our knowledge of the formation, storage and depletion of carbohydrates in monocotyledons. *Philos. Trans.* B, **191**, 35–79.

Rosing, M. S. (1908). Der Zucker- und Stärkegehalt in den Schleisszellen offener und geschlossener Spaltöffnungen. *Ber. dtsch. bot. Ges.* **26***a*, 438–45.

Sayre, J. D. (1923). Physiology of stomata of *Rumex patientia*. *Science*, **57**, 205–6.

Sayre, J. D. (1926). Physiology of stomata of *Rumex patientia*. *Ohio J. Sci.* **26**, 233–66.

Scarth, G. W. (1929). The influence of H-ion concentration on the turgor and movement of plant cells with special reference to stomatal behaviour. *Proc. Int. Cong. Pl. Sci., Ithaca*, pp. 1151–62.

Scarth, G. W. (1932). Mechanism of the action of light and other factors on stomatal behaviour. *Plant Physiol.* **7**, 481–504.

Schwendener, S. (1881). Über Bau und Mechanik der Spaltöffnungen. *Monatsber. Berl. Akad.* pp. 833–67.

Semmens, E. S. (1947). Starch hydrolysis induced by polarized light in stomatal guard cells of living plants. *Plant Physiol.* **22**, 270–8.

Small, J., Clarke, M. I. & Crosbie-Baird, J. (1942). pH phenomena in relation to stomatal opening. II–V. *Proc. Roy. Soc. Edin.* B, **61**, 233–66.

Small, J. & Maxwell, K. M. (1939). pH phenomena in relation to stomatal opening. I. *Coffea arabica* and some other species. *Protoplasma*, **32**, 272–88.

Steinberger, A. L. (1922). Regulation des osmotischen Wertes in den Schliesszellen. *Biol. Zbl.* **42**, 405–19.

Strugger, S. & Weber, F. (1926). Zur Physiologie der Stomatanebenzellen. *Ber. dtsch. bot. Ges.* **44**, 272–8.

Weber, F. (1923). Enzymatische Regulation der Spaltöffnungsbewegung. *Naturwiss.* **11**, 309–16.

Wiggans, R. G. (1921). Osmotic concentration of the guard cells. *Amer. J. Bot.* **8**, 30–40.

Williams, W. T. (1948). Studies in stomatal behaviour. I. Stomatal movement induced by heat-shock stimuli, and the transmission of such stimuli across the leaves of *Pelargonium zonale*. *Ann. Bot., Lond.*, **12**, 35–51.

EXPLANATION OF PLATES 1–10

PLATE 1

Experiment of 25 July 1938. Epidermal strips taken by Lloyd's method. Mounted in iodine-phenol-KI reagent to show stomatal starch contents and apertures. 8 mm. apochromat and 10 ×. Periplanatic eyepiece. For magnification, see Pl. 8, fig. 7.

Fig. 9. Strip no. 1.

Fig. 10. Strip no. 3.

Fig. 11. Strip no. 6.

For details, strips nos. 1–6, see Table 3, p. 199.

PLATE 2

Exp. 12. Technique as for Pl. 1.
Fig. 1. Plant A in light, 10.5 a.m., 13 June 1944.
Fig. 2. Plant A in darkness, 10.10 a.m., 14 June 1944.
Fig. 3. Plant B in darkness, 10.10 a.m., 13 June 1944.

PLATE 3

Exps. 12 and 30. Technique as for Pl. 1.
Fig. 4. Exp. 12. Plant B in light, 10.5 a.m., 14 June 1944.
Fig. 5. Exp. 30. Plant A in darkness, 10.50 a.m., 28 June 1945.
Fig. 6. Exp. 30. Plant A in light, 10.45 a.m., 29 June 1945.

PLATE 4

Experiment of 13–14 October 1938. Technique as for Pl. 1.
Fig. 19. Strip no. 44.
Fig. 20. Strip no. 45.
Fig. 21. Strip no. 46.
For details, strips nos. 44–46, see Table 4, p. 200.

PLATE 5

Experiment of 24–25 October 1939. Technique as for Pl. 1.
Fig. 30. Strip no. 81.
Fig. 31. Strip no. 84.
Fig. 32. Strip no. 86.
For details, strips nos. 81–86, see Table 5, p. 201.

PLATE 6

Experiments of 24–25 October 1939 and 16–17 November 1939. Technique as for Pl. 1.
Fig. 33. Strip no. 89. For details see Table 5, p. 201.
Fig. 43. Strip no. 139.
Fig. 44. Strip no. 143.
For details, strips nos. 139–143, see Table 6, p. 202.

PLATE 7

Experiment of 16–17 November 1939. Technique as for Pl. 1.
Fig. 45. Strip no. 144.
Fig. 46. Strip no. 145.
Fig. 47. Strip no. 146.
For details, strips nos. 144–146, see Table 6, p. 202.

PLATE 8

Experiments of 25 July 1938 and 13–14 October 1938. Epidermal strips taken by Lloyd's method. Mounted in iodine-phenol-KI reagent to show stomatal starch content and aperture. $\frac{1}{7}$ in. oil immersion achromat and 10 × Periplanatic eyepiece. For magnification, see fig. 8. First column, stomata with maximal starch; second column, stomata with minimal starch; third column, stomata with medium starch contents, also stage micrometer photographed to show magnification of low-power (fig. 7) and high-power (fig. 8) photomicrographs on all plates.

Fig. 7. Stage micrometer, 10 μ divisions, photographed with optical system as for Pls. 1–7.
Fig. 8. Ditto, photographed with optical system as for Pls. 8–10.
Fig. 12. Strip no. 1. Maximal starch.
Fig. 13. Strip no. 1. Minimal starch.
Fig. 14. Strip no. 3. Maximal starch.
Fig. 15. Strip no. 3. Minimal starch.
Fig. 16. Strip no. 3. Medium starch.
Fig. 17. Strip no. 6. Maximal starch.
Fig. 18. Strip no. 6. Minimal starch.
For details, strips nos. 1–6, see Table 3, p. 199.
Fig. 22. Strip no. 44. Maximal starch.
Fig. 23. Strip no. 44. Minimal starch.
Fig. 25. Strip no. 45. Medium starch.
For details, strips nos. 44–45, see Table 4, p. 200.

PLATE 9

Experiments of 13–14 October 1938 and 24–25 October 1939. Technique as for Pl. 8. First column, stomata with maximal starch; second column, stomata with minimal starch; third column, various.

Fig. 24. Strip no. 45. Minimal starch.
Fig. 26. Strip no. 46. Maximal starch.
Fig. 27. Strip no. 46. Minimal starch.
Fig. 28. Strip no. 50. Maximal starch.
Fig. 29. Strip no. 50. Minimal starch.
For details, strips nos. 45–50, see Table 4, p. 200.
Fig. 34. Strip no. 81. Maximal starch.
Fig. 35. Strip no. 81. Minimal starch.
Fig. 36. Strip no. 84. Maximal starch.
Fig. 37. Strip no. 84. Minimal starch.
Fig. 38. Strip no. 86. Maximal starch.
Fig. 39. Strip no. 86. Minimal starch.
Fig. 40. Strip no. 86. Medium starch.
For details, strips nos. 81–86, see Table 5, p. 201.

PLATE 10

Experiments of 24–25 October 1939 and 16–17 November 1939. Technique as for Pl. 8. First column, stomata with maximal starch; second column, stomata with minimal starch; third column, stomata with maximal and minimal starch.

Fig. 41. Strip no. 89. Maximal starch.
Fig. 42. Strip no. 89. Minimal starch.
For details, strip no. 89, see Table 5, p. 201.
Fig. 48. Strip no. 139. Maximal starch.
Fig. 49. Strip no. 139. Minimal starch.
Fig. 50. Strip no. 143. Maximal starch.
Fig. 51. Strip no. 143. Minimal starch.
Fig. 52. Strip no. 144. Maximal starch.
Fig. 53. Strip no. 144. Minimal starch.
Fig. 54. Strip no. 145. Maximal starch.
Fig. 55. Strip no. 145. Minimal starch.
Fig. 56. Strip no. 146. Maximal starch.
Fig. 57. Strip no. 146. Minimal starch.
For details, strips nos. 139–146, see Table 6, p. 202.

MULTIPLE-ALLELOMORPH HETEROTHALLISM IN THE FUNGI

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(With 2 figures in the text)

CONTENTS

PAGE

1. Introduction 212
2. The distribution of the occurrence of multiple-allelomorph heterothallism 217
3. The relative frequencies of homothallic and of bipolar and tetrapolar heterothallic species . 218
4. The number of allelomorphs per fruit-body 219
5. Mutation at the loci for heterothallism 220
6. The distribution of the allelomorphs at the loci for heterothallism in natural populations of species 222
7. The number of allelomorphs at the loci for heterothallism in natural populations of species . 225
8. Location of the two loci for heterothallism in tetrapolar species 228
    (*a*) Linkage data 228
    (*b*) Data from the analysis of tetrads 230
9. List of homothallic and of bipolar and tetrapolar heterothallic species of Hymenomycetes and Gasteromycetes 233
10. Summary 239

References 240

I. INTRODUCTION

Heterothallism in the Hymenomycetes was first reported by Mlle. Bensaude (1917) to the Académie des Sciences of Paris, and published fully the following year (Bensaude, 1918). Bensaude discovered that in *Coprinus fimetarius* the clamp-connexions are associated with the simultaneous or 'conjugate' division of a pair of nuclei. She found that single-spore cultures, unlike cultures derived from many spores, did not produce clamp-connexions or fruit-bodies. Furthermore, she obtained two cultures, each originating from a single spore, which separately remained in the primary condition indefinitely, but when placed together they gave rise to hyphae with clamp-connexions, followed by the formation of fruit-bodies. Bensaude regarded the two strains as analogous to the plus and minus strains of the heterothallic species of the Mucorales.

The association of clamp-connexions with the simultaneous division of two nuclei had already been discovered in Germany by Kniep (1915) in *Corticium varians* and *C. serum*, but on account of the war Bensaude did not know of this work until 1918. At a meeting of the Physikalisch-medizinischen Gesellschaft of Würzburg in November 1919, Kniep (1920) reported the discovery of heterothallism in *Schizophyllum commune* and a number of other Hymenomycetes. Kniep did not know of Bensaude's work at this time, and had independently discovered heterothallism by following up experiments made by Miss Wakefield (1909). Certain single-spore cultures of *S. commune* had been found to produce fruit-bodies, and others not. Kniep confirmed this, and further found that none of the single-spore cultures produced clamp-connexions, but that when the cultures were combined in pairs, certain combinations produced clamps. By cytological studies he was able to show that in the basidia of the fruit-bodies produced in single-spore cultures, nuclear fusion did not occur and meiosis was replaced by mitosis. When the single-spore cultures of *S. commune* were paired in all combinations, Kniep found that they fell into four groups according to their behaviour in forming clamp-connexions. If these four groups are numbered I, II, III and IV, then, with a few exceptions, any single-spore culture in group I formed clamp-connexions with any single-spore culture in group II, and any culture in group III formed clamps with any in group IV, but all other combinations of cultures in pairs failed to produce clamps. To explain this result he suggested that two pairs of factors were segregating independently; he called the one pair **A** and **a**, and the other **B** and **b**, and suggested that conjugate nuclear division, indicated by the formation of clamp-connexions, could only take place if the two nuclei that became associated, differed in both their **A** and their **B** factors. Classes I, II, III and IV above would then correspond to the constitutions **AB**, **ab**, **Ab** and **aB**, respectively. The diploid nucleus in each basidium would have the constitution **AaBb**, formed by the fusion either of nuclei **AB** and **ab**, or **Ab** and **aB**. Kniep also discovered that when the single-spore cultures from one fruit-body of *S. commune* were paired with those of another fruit-body from a different source, clamp-connexions were formed in every pairing. It was thus clear that heterothallism in *S. commune* was of a different character from that of the Mucorales, and also from that of *Ustilago violacea*, in which, earlier that year, Kniep had reported the discovery of heterothallism of a simple type with only two strains (Kniep, 1919).

Lehfeldt (1923), working under Kniep at Würzburg, was the first to observe the actual fusion of hyphae when two single-spore cultures of different mating types meet. He worked with *Corticium serum* and *Typhula erythropus*, which Kniep had already shown to be heterothallic species. Lehfeldt demonstrated that after the hyphal fusion, nuclear migration takes place, having been made possible by the partial breakdown of the cross-walls of the hyphae. In *Corticium serum* he found that the first clamp-connexion and its associated conjugate nuclei appeared close to the point of the hyphal fusion and shortly afterwards, but in *Typhula erythropus* the appearance of the first clamp was somewhat delayed and not associated so directly with the position of the hyphal fusion.

Meanwhile, Bensaude's discovery of heterothallism in *Coprinus fimetarius* had aroused widespread interest. Research along similar lines was begun by Lendner at Geneva, by Miss Mounce at Winnipeg, and by Vandendries at Antwerp. Lendner (1920) obtained six single-spore cultures of *C. clavatus* and showed that fruit-bodies were formed only in certain paired cultures, thus demonstrating that this species is heterothallic. He also showed that *C. sterquilinus* is homothallic by the criterion of fruit-body formation in single-spore culture, though Kniep's discovery of haploid fruit-body formation in single-spore cultures of *Schizophyllum commune*, which was unknown to Lendner, somewhat invalidates this criterion.

Mounce (1921, 1922) obtained single-spore cultures of a number of species of *Coprinus* and showed that *C. sterquilinus* and *C. stercorarius* are homothallic on the basis of the formation of clamp-connexions and fruit-bodies in single-spore cultures, and *C. comatus*, *C. lagopus* and *C. niveus* heterothallic. After learning of Kniep's discovery that *Schizophyllum commune* is 'tetrapolar', i.e. with spores of four different mating types on each fruit-body, she tested *Coprinus lagopus* and *C. niveus* and found they were tetrapolar, also. This was of special interest, since Buller considered Mounce's *C. lagopus* to be identical with Bensaude's *C. fimetarius*.

Vandendries (1922, 1923*a*), following Bensaude's method, was able to show that *Hypholoma fasciculare*, *Collybia velutipes*, *Anellaria separata* (L.) Karst. [syn. *Panaeolus separatus* (L.) Fr.], *Panaeolus campanulatus* and *P. fimicola* were all heterothallic, on the basis of the absence of clamp-connexions in single-spore cultures and their presence in certain paired cultures.

Meanwhile, Kniep (1922) demonstrated the existence of multiple allelomorphs of the factors for heterothallism in *Schizophyllum commune*. He paired together single-spore cultures of two fruit-bodies from different sources, and from the resulting hybrid fruit-bodies he obtained a new set of single-spore cultures. He found that when these were paired in all combinations they showed the existence of spores of four different mating types, just like the wild fruit-bodies. Kniep showed that if one of the original wild fruit-bodies had spores of constitution **AB**, **ab**, **Ab**, and **aB**, and the other of constitution **CD**, **cd**, **Cd** and **cD**, then when a single-spore culture of one (say **AB**) was crossed with a single-spore culture of the other (say **CD**), the resulting fruit-bodies produced spores of constitutions **AB**, **CD**, **AD** and **BC**. In other words, there was no recombination between **A** and **C**, or between **B** and **D**. As **A**, **a**, **C** and **c** are thus all allelomorphs at one locus they are best represented as $\mathbf{a}_1$, $\mathbf{a}_2$, $\mathbf{a}_3$ and $\mathbf{a}_4$ respectively and, similarly, **B**, **b**, **D** and **d** as $\mathbf{b}_1$, $\mathbf{b}_2$, $\mathbf{b}_3$ and $\mathbf{b}_4$ respectively.

Kniep (1922) also investigated individual tetrads of spores, making use of the fact that in *Aleurodiscus polygonius*, under certain conditions, the four spores from a basidium are shot away together. Having isolated individual tetrads, he separated the spores by shaking them in a broth culture, and in this way obtained single-spore cultures of the four spores from each of thirty-five basidia. He found that *A. polygonius* is tetrapolar like *Schizophyllum commune*, and that in every instance there were spores of only two mating types on each basidium, either two $\mathbf{a}_1\ \mathbf{b}_1$ and two $\mathbf{a}_2\ \mathbf{b}_2$, or two $\mathbf{a}_1\ \mathbf{b}_2$ and two $\mathbf{a}_2\ \mathbf{b}_1$; he concluded that segregation of factors occurred at the first division of meiosis. He also showed, by the analysis of nearly 300 single-spore cultures, that there was no linkage between the *a* and *b* loci in *Aleurodiscus polygonius*.

The following year, Kniep (1923) published evidence for the occurrence of frequent mutations at both the *a* and *b* loci of *Schizophyllum commune*, with the production of new allelomorphs. He also obtained fruit-bodies of this species from twelve different sources and found that they all contained a different pair of allelomorphs at each locus.

The occurrence in the Hymenomycetes of 'bipolar' heterothallism, i.e. spores of only two mating types on each fruit-body, was discovered independently by Vandendries (1923*b*, 1924), Brunswik (1923, 1924) and Miss Newton (see Buller, 1924*b*). In a paper submitted for publication in July 1923, Vandendries (1923*b*) reported that *Anellaria separata* (L.) Karst. [syn. *Panaeolus separatus* (L.) Fr.] was bipolar with only two classes of spores on a fruit-body. He also found that in *P. campanulatus* there was complete fertility between single-spore cultures of three fruit-bodies from different sources. In *Coprinus radians* he found another example of a bipolar species, and here also he showed there was complete fertility between single-spore cultures of three fruit-bodies from different sources (Vandendries, 1924, 1925*a–c*). He also claimed to have found evidence for mutation at the locus for heterothallism in this species.

In October 1923, at a meeting of the Zoologisch-botanischen Gesellschaft of Vienna, Brunswik (1923) reported a detailed investigation of heterothallism and homothallism in the genus *Coprinus*. He had found that some of the heterothallic species were bipolar and some tetrapolar, and that in either case single-spore cultures of one fruit-body showed fertility in all or almost all pairings with those of another fruit-body from a different source. This work was reported in full the following year (Brunswik, 1924). In the bipolar species *C. comatus*, he found the same allelomorph appeared in three fruit-bodies from widely separated localities, but in the tetrapolar species *C. fimetarius* more than a dozen fruit-bodies from different localities all showed different allelomorphs at both loci.

Newton, as reported by Buller (1924*b*), had found independently of Vandendries and Brunswik that *C. rostrupianus* was bipolar (Newton, 1926*a*).

The occurrence of tetrapolar heterothallism with multiple allelomorphs, discovered by Kniep (1920, 1922) in *Schizophyllum commune*, was confirmed by Brunswik (1924) in *Coprinus fimetarius*, by Hanna (1925) in *C. lagopus*, and by Vandendries (1926) in *C. micaceus*. Thus by 1926 it had become generally established that there were both bipolar and tetrapolar species among heterothallic Hymenomycetes, and that both types had multiple allelomorphs at the loci responsible for the heterothallism. Since that time the number of species in which heterothallism has been investigated has been much increased, due particularly to the researches of Vandendries and Quintanilha.

In tetrapolar species, the occurrence of four spores, all of different mating types on a single basidium, was discovered independently by Funke working under Kniep at Würzburg, and by Hanna working under Buller at Winnipeg. Funke (1924) succeeded in isolating by means of a micromanipulator all four spores from a number of basidia of *Hypholoma fasciculare*, *H. capnoides* and *Collybia velutipes*. He found that all three species were tetrapolar and, in each case, in addition to basidia bearing spores of only two mating types, there were others with four different mating types present ($\mathbf{a}_1\,\mathbf{b}_1$, $\mathbf{a}_2\,\mathbf{b}_2$, $\mathbf{a}_1\,\mathbf{b}_2$ and $\mathbf{a}_2\,\mathbf{b}_1$). He concluded that in these basidia segregation took place at the second division of meiosis.

Hanna, as first reported by Buller (1924*b*), obtained a similar result with *Coprinus lagopus* (Hanna, 1925). He removed a gill from a young fruit-body and placed a cover-slip against one surface. He found that the tetrads of spores from the individual basidia frequently adhered to the cover-slip in the form of a square, and he was able to remove the spores separately with a dry needle. Further analyses of the spores of individual basidia of *C. lagopus* were made by Newton (1926*b*), using Hanna's method.

This discovery by Funke and Hanna of spores of four different mating types on a proportion of the basidia in tetrapolar species was confirmed by Kniep in *Aleurodiscus polygonius* (see Newton, 1926*a*, *b*; Kniep, 1928), and by Brunswik (1926), who isolated the spores of ninety-three basidia of *Coprinus fimetarius*.

The spores of individual basidia of bipolar species were isolated, using Hanna's method, by Newton (1926*a*) working with *C. rostrupianus*, and by Vandendries (1925*c*) with *C. radians*. Each basidium was found to bear two spores of one mating type and two of another.

Haploid fruit-bodies, such as Kniep (1920) had discovered in *Schizophyllum commune*, have been found to occur in many species of Hymenomycetes when single-spore cultures are maintained in the primary condition (see Buller, 1941). This means that the formation of fruit-bodies is not a reliable criterion for separating homothallic and heterothallic species. However, the presence of clamp-connexions in single-spore cultures constitutes satisfactory evidence for homothallism; and the absence of clamps in single-spore cultures and their appearance when certain single-spore cultures are paired, have formed the basis for the determination of heterothallism in the Hymenomycetes, and for the separation of bipolar and tetrapolar species.

The homothallism of *Coprinus sterquilinus* was investigated by Harder (1926). He showed that uninucleate hyphal tips, if severed from the rest of the mycelium and isolated, were capable of growing and giving rise to new hyphae with clamp-connexions. He thus established that in this species sister nuclei can become associated in pairs, and divide conjugately with the formation of clamp-connexions.

Some species of Hymenomycetes which appear homothallic are basically heterothallic, since two nuclei of compatible mating types are included in each basidiospore before it is set free from the basidium. Such a secondarily homothallic species is *Aleurodiscus canadensis*, described by Skolko (1944). This species has only two spores on each basidium, and each spore receives two of the four nuclei resulting from meiosis. Secondarily homothallic two-spored forms of heterothallic four-spored species were first described by Sass (1929) for *Coprinus ephemerus*, *Galera tenera* and *Naucoria semiorbicularis*. Oikawa (1939) showed that *Galera tenera* is tetrapolar. If the two-spored secondarily homothallic form is also tetrapolar, in those basidia in which all four nuclei, after meiosis, are of different mating types, the $\mathbf{a}_1\,\mathbf{b}_1$ and $\mathbf{a}_2\,\mathbf{b}_2$ nuclei presumably pass into one spore and the $\mathbf{a}_1\,\mathbf{b}_2$ and $\mathbf{a}_2\,\mathbf{b}_1$ nuclei into the other. Any other grouping in pairs of the four nuclei would give incompatible combinations.

Knowledge of the distribution of multiple-allelomorph heterothallism in the Fungi has been widened by the work of Barnett (1937) on the Tremellales, and of Fries (1940) on the Nidulariales (Gasteromycetes). Detailed studies have been made by Fries of the frequency and distribution of different allelomorphs at the loci for heterothallism in the populations of several tetrapolar species of Hymenomycetes and Gasteromycetes (Fries, 1940, 1943; Fries & Jonasson, 1941).

The misleading term 'geographical races' was adopted by Brunswik (1924) and Kniep (1928) for individuals of heterothallic Hymenomycetes from different sources, which showed complete fertility when single-spore cultures were paired, and which thus merely differed in the allelomorphs they carried. Vandendries (1929*a*) emphasized the inappropriateness of this term, after finding true intersterile geographical races in *Coprinus micaceus* (Vandendries, 1927*a–c*; Vandendries & Robyn, 1929); and Mather (1942) has likewise criticized its use for fully interfertile strains. The analogy between multiple-allelomorph heterothallism in the Hymenomycetes, and self-sterility in flowering plants, was pointed out by Kniep (1922) and Brunswik (1924). Kniep, however, insisted that the genes for heterothallism in the Hymenomycetes were essentially sex factors, whereas Brunswik regarded them merely as self-sterility factors and so drew a closer analogy with incompatibility in flowering plants. The discovery that this incompatibility of pollen and style usually has as its genetic basis a multiple allelomorphic series at one locus supports the analogy, as Mather (1942) has pointed out. Furthermore, Mather has shown that the second locus of the tetrapolar species can be regarded as an adaptation which promotes out-breeding by decreasing the frequency of fertile sister-matings, while multiple allelomorphs also favour out-breeding, but act by increasing the frequency of fertile non-sister matings.

2. THE DISTRIBUTION OF THE OCCURRENCE OF MULTIPLE-ALLELOMORPH HETEROTHALLISM

Since the discovery by Kniep (1920) that there was complete fertility in all pairings of single-spore cultures of one fruit-body of *Schizophyllum commune* with those of another from a different source, a similar condition has been found in a considerable number of other heterothallic species of Hymenomycetes and in a few Gasteromycetes. In comparatively few instances has any attempt been made to determine whether this complete cross-fertility originates from multiple allelomorphs at the loci for heterothallism such as Kniep (1922) had demonstrated in *Schizophyllum commune*. Cross-breeding experiments with strains from different fruit-bodies, which have shown the existence of such multiple allelomorphs, have been made with the following tetrapolar species: *Coprinus fimetarius* (Brunswik, 1924); *C. lagopus* (Hanna, 1925); *Cyathus striatus* (Fries, 1940);*Polyporus abietinus* (Fries & Jonasson, 1941); *Panus stypticus* (Macrae, 1942); and *Crucibulum vulgare* (Fries, 1943). No similar investigations appear to have been made in bipolar species, and in them the evidence for the existence of multiple allelomorphs, apart from the analogy with tetrapolar species, rests primarily upon the rare occurrence of clamp-connexions in only three-quarters of the combinations when single-spore cultures of fruit-bodies from different sources are paired, implying that the two fruit-bodies had an allelomorph in common. Such an occurrence was found twice by Brunswik (1924) in *Coprinus comatus* and once by Mounce & Macrae (1937) in *Fomes roseus*. Thus, it appears very probable that there are multiple allelomorphs at the loci for heterothallism in all those species of Hymenomycetes and Gasteromycetes, whether bipolar or tetrapolar, in which cross-fertility of strains from different sources has been demonstrated. Accordingly, in the list in section 9, all those species in which cross-fertility of single-spore cultures of fruit-bodies from different sources has been demonstrated, are assumed to have multiple allelomorphs at the loci for heterothallism, irrespective of the existence or not of definite proof by cross-breeding experiments or other evidence.

Of the 207 heterothallic species of Hymenomycetes and Gasteromycetes listed in section 9, there is evidence (on the basis of cross-fertility) for the existence of multiple allelomorphs at the loci for heterothallism in at least sixty of them. In the remainder, no tests appear to have been made. Thus, wherever multiple allelomorphs have been looked for in heterothallic Hymenomycetes and Gasteromycetes they have been found, and since they have been shown to occur in sixty species, it is likely that all the heterothallic species possess multiple allelomorphs. Barnett (1937) showed that *Auricularia auricula-judae* in the Auriculariaceae and several species of *Exidia* in the Tremellaceae possess multiple-allelomorph heterothallism, thus establishing its occurrence in the order Tremellales of the Hymenomycetes; and Fries (1940, 1943) showed that it occurs in *Cyathus striatus* and *Crucibulum vulgare* in the order Nidulariales of the Gasteromycetes. It probably occurs also in the other four orders of Gasteromycetes, but in these no tests appear to have been made.

Multiple-allelomorph heterothallism has not been recorded in the Phycomycetes or the Ascomycetes, and in many genera of these classes such as *Mucor* and *Neurospora*, interspecific hybridization and crosses between individuals from different sources have established that there are only two allelomorphs at the loci for heterothallism. In the Uredinales, likewise, there is no record of multiple allelomorphs for heterothallism, and in a number of species there is good evidence for the existence of only two allelomorphs. In the Ustilaginales numerous interspecific crosses have been made,. and it it well established that in the majority of the heterothallic species there is only one locus with two allelomorphs. In certain species, notably *Ustilago maydis* (DC.) Corda [syn. *U. zeae* (Beckm.) Unger], tetrapolar heterothallism has been claimed (Hanna, 1929; Christensen, 1929, 1932), but there has been no suggestion of the existence of multiple allelomorphs. In *U. longissima* (Sow.) Tul., on the other hand, Bauch (1930, 1931) claimed to have found two loci each with multiple allelomorphs. A critical study of Hanna and Christensen's work on *U. maydis* and Bauch's work on *U. longissima* suggests that in these species, as in other smut fungi, heterothallism is controlled by one locus with two allelomorphs.* Whether *U. longissima* is exceptional or not, it is certainly true that in the majority of the species of Ustilaginales that have been investigated multiple-allelomorph heterothallism does not occur.

From existing knowledge of heterothallism in the Fungi, it seems probable that multiple-allelomorph heterothallism is a characteristic of the Hymenomycetes (including the Tremellales) and the Gasteromycetes, and that multiple allelomorphs at the loci for heterothallism are found only in these two subclasses of Basidiomycetes and nowhere else in the Fungi.

Outside the Fungi, incompatibility of pollen and style due to multiple allelomorphs at one locus, appears to be of widespread occurrence in the flowering plants. In the ciliated Protozoa, multiple interbreeding mating types are known in *Paramecium bursaria* and, of a different kind, in *Euplotes patella* (see Sonneborn, 1947). In the wasp, *Habrobracon juglandis*, there is evidence for sex determination by a series of multiple allelomorphs at one locus (Whiting, 1943). The occurrence of two loci, however, each with multiple allelomorphs, as the basis of an out-breeding mechanism, does not appear to have been recorded outside the Fungi, apart from certain instances of incompatibility in allotetraploid flowering plants.

3. THE RELATIVE FREQUENCIES OF HOMOTHALLIC AND OF BIPOLAR AND TETRAPOLAR HETEROTHALLIC SPECIES

The list of homothallic and heterothallic species of Hymenomycetes and Gasteromycetes given in section 9 includes those listed by Kniep (1920, 1922, 1928), Vandendries (1934*d*), Biggs (1938), Quintanilha (1941) and Langeron (1945), as well as many others. Out of a total of 230 species listed, 207 or 90% are recorded as being heterothallic and 23 or 10% as homothallic. In the case of *Odontia fusco-atra* and *Coprinus stercorarius*, different workers have differed in their findings, and so it has been assumed that these species are really complexes of separate homothallic and heterothallic species, and they have been recorded in both categories. An analogous condition was found by Biggs (1937) for *Corticium coronilla*, and in this instance three species have been recognized (one homothallic, one heterothallic and bipolar, and one heterothallic and tetrapolar).

Of the 207 heterothallic species, 168 have been investigated in more detail and they comprise sixty-four bipolar species (with one locus for heterothallism) and ninety-nine tetrapolar species (with two loci). These figures include certain species twice, since besides the case of *C. coronilla* already referred to, the following species have been recorded as bipolar by one person and tetrapolar by another: *Anellaria separata*, *Coprinus curtus*,

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* A detailed analysis of published work on heterothallism in the Ustilaginales will be published elsewhere.

C. macrorhizus, *C. radians* and *Psilocybe coprophila*. If has therefore been assumed that each of these taxonomic species is really a complex of at least two natural species, and they have been recorded as such. The secondarily homothallic species *Calocera cornea* and *Aleurodiscus canadensis* have been counted as heterothallic, since they have genes for heterothallism. It would thus appear that approximately 39% of the heterothallic species are bipolar and 61% tetrapolar.

Taking the Hymenomycetes and Gasteromycetes as a whole, the data obtained from the sample of species that has been investigated, suggest that approximately 10% are homothallic, 35% heterothallic and bipolar, and 55% heterothallic and tetrapolar. These figures take no account of apomictic forms with two-spored basidia, where there is no nuclear fusion in the basidium; nor do they include two-spored secondarily homothallic forms of four-spored heterothallic species (see Sass, 1929; Smith, 1934; Vandendries, 1937*c*; Kühner, 1938). Insufficient appears to be known at present about most of these two-spored forms, either asexual or secondarily homothallic, to establish their true taxonomic position. In some instances it seems possible that the asexual forms may be merely isolated strains of one mating type of heterothallic species, while with the two-spored secondarily homothallic forms it is not known whether or not they are cross-fertile with the corresponding four-spored heterothallic species.

An analysis of the relative frequencies of homothallic and of bipolar and tetrapolar heterothallic species in the different groups of Hymenomycetes and Gasteromycetes suggests that there may be no significant differences between orders, families or genera, but the data are in many cases quite inadequate for a precise statement. Thus, no tetrapolar Tremellales, no homothallic Polyporaceae and no homothallic or bipolar Nidulariales have been recorded, but these may all be due to chance.

4. THE NUMBER OF ALLELOMORPHS PER FRUIT-BODY

Brefeld (1877) described the stages of development of a fruit-body in *Coprinus stercorarius*, *C. lagopus*, *C. ephemerus*, *C. ephemeroides*, *Amanita muscaria*, and other species. He showed that development began from a single cell of the mycelium. With the discovery by Kniep (1915) and Bensaude (1918) that clamp-connexions are associated with the simultaneous division of a pair of nuclei, it became evident that if a fruit-body originated from a single cell, the nuclei in all the cells of the hyphae and in all the basidia of that fruit-body were probably daughter-nuclei of the original pair in the first cell. Remarkable confirmation of this has come from genetical studies of Hymenomycetes and Gasteromycetes, since the discovery by Kniep (1920, 1922) of multiple allelomorphs at the loci for heterothallism. In all the investigations of the pairing reactions of single-spore cultures, there does not appear to be a single example of more than two allelomorphs having been recorded at one locus amongst the spores from one fruit-body, apart from instances of mutation (see section 5). This applies to all the 163 bipolar and tetrapolar species listed in section 9. It implies that the hymenium of each fruit-body, if not the sterile tissue as well, invariably arises from or can ultimately be traced back to a single cell containing a pair of nuclei.

The only laboratory investigations that have been made to test the hypothesis that fruit-bodies do not normally contain more than two allelomorphs at one locus appear to be those of Brunswik (1924) for *Coprinus fimetarius*, and of Oikawa (1939) for *Stropharia semiglobata* and *Galera tenera*. Brunswik inoculated a culture medium with three, or in some experiments four, different mycelia derived from single spores of the tetrapolar species *Coprinus fimetarius*. Each mycelium carried a different allelomorph at each locus. He identified the allelomorphs present in the spores from the resulting fruit-bodies, and in no case did he find more than two allelomorphs at one locus amongst the spores from any one fruit-body, although different fruit-bodies from the same culture showed different pairs of allelomorphs. It was thus clear that one binucleate cell initiated the formation of the hymenium if not of the entire structure of each fruit-body. Brunswik could offer no satisfactory explanation for the combination of allelomorphs which he found in certain of the fruit-bodies. However, he overlooked the fact that his findings can be explained quite simply if one of the haploid cultures, say $\mathbf{a}_1\, \mathbf{b}_1$, contained, in addition, nuclei of constitution $\mathbf{a}_2\, \mathbf{b}_1$, which would be incompatible with $\mathbf{a}_1\, \mathbf{b}_1$, and so would not give rise to clamp-connexions and hence would pass undetected.

Oikawa (1939) made a similar series of experiments with the bipolar species *Stropharia semiglobata* and the tetrapolar species *Galera tenera*, and found that in both species the number of allelomorphs per locus was strictly confined to two amongst the spores from one fruit-body, even though three or four allelomorphs were present in the culture.

Buller (1931, pp. 171, 172) considers that single fruit-bodies of *Coprinus lagopus** and other heterothallic Hymenomycetes are not always the product merely of the two mycelia of opposite mating type from which their nuclei are derived, but are often the product of a compound mycelium made up of a few to many thousands of mycelia. It is probably true that a large number of different allelomorphs at the loci for heterothallism are often present in the nuclei of the mycelium of fungi such as *C. lagopus* in nature (see section 6), but all the evidence, including Buller's own experiments, points to the conclusion that individual fruit-bodies normally arise from a single cell containing a pair of nuclei of different mating types.

Exception must be made of the haploid apomictic fruit-bodies formed by many species of Hymenomycetes when grown in single-spore culture. In these, only a single allelomorph can be present at each locus. Exception must also be made of fruit-bodies of tetrapolar species obtained in culture by mating single-spore mycelia which have an allelomorph in common at one locus but not at the other, e.g. $\mathbf{a}_1\, \mathbf{b}_1$ and $\mathbf{a}_1\, \mathbf{b}_2$. These have been studied in *C. fimetarius* by Oort (1930), Buller (1931) and Quintanilha (1935, 1937, 1941). It appears uncertain whether such fruit-bodies are apomictic or functionally bipolar, but in either case only one allelomorph is present at one of the loci.

Thus, all the evidence suggests that fruit-bodies of heterothallic species of Hymenomycetes and Gasteromycetes normally have two allelomorphs at each locus for heterothallism, and arise from a single cell containing a pair of nuclei of different mating types.

5. MUTATION AT THE LOCI FOR HETEROTHALLISM

Soon after the discovery of multiple-allelomorph heterothallism in the fungi, Kniep (1923) produced evidence for the occurrence of frequent mutations at the loci for heterothallism, with the production of new allelomorphs. Perhaps as a consequence of this, many subsequent workers have readily accepted mutation as the explanation of any irregularities in their tables of pairing reactions of single-spore cultures, without attempting to eliminate alternative explanations, and the idea has thus grown up that mutation is a frequent occurrence at the loci for heterothallism in the Hymenomycetes. When the evidence is examined critically, it is found that in a majority of cases proof of mutation is lacking. The only instances of mutation that appear to be well substantiated are the original claim by Kniep (1923) in *Schizophyllum commune* and those of Zattler (1924) in *Collybia velutipes* and Quintanilha (1935) in *Coprinus fimetarius*.

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* *C. lagopus* Fr. sensu Buller = *C. fimetarius* (L.) Fr. See Buller (1931, pp. 190–3).

Kniep (1923) paired together two single-spore cultures of the tetrapolar species *Schizophyllum commune*, from fruit-bodies from different localities, and isolated nineteen single spores from a resulting fruit-body. Sixteen of these fell into the expected four classes as regards the formation of clamp-connexions when paired together, but the other three single-spore cultures showed clamp-formation with two of the four classes instead of only one. By breeding experiments, Kniep showed that these three cultures were mutants, two of them carrying different new allelomorphs at the *a* locus and the third a new allelomorph at the *b* locus. That three different mutations should be present amongst so few as nineteen spores from one fruit-body was remarkable. Kniep (1928, 1930) continued breeding experiments with *S. commune* for twenty-four generations, and claimed that further mutations appeared sporadically in his cultures, though often for several successive generations there were none.

Zattler (1924, pp. 470–5) obtained results with *Collybia velutipes* similar to those of Kniep with *Schizophyllum commune*. Zattler, like Kniep, tested to make sure that his mutants were not mixed mycelia containing incompatible nuclei such as $\mathbf{a}_1\,\mathbf{b}_1$ and $\mathbf{a}_1\,\mathbf{b}_2$. These results were discussed by Kniep (1929).

Quintanilha (1935) obtained several spontaneous mutations in *Coprinus fimetarius* and made adequate tests to check that they were indeed new allelomorphs. However, neither Kniep, Zattler, nor Quintanilha, reported having tested the mutant allelomorphs against other allelomorphs of the same species which they had in their laboratories. If they had demonstrated that their mutant allelomorphs were different from any of those that they had in culture, then any slight doubt that the 'mutants' were not really due to mutation but to stray spores from other fruit-bodies, would have been removed.

The claims of Vandendries, Hanna, Newton and Brunswik to have found mutations are much less convincing. Vandendries (1924, 1925*a–c*) claimed to have demonstrated mutation in the bipolar species *C. radians*. He isolated twenty-three single spores from one fruit-body and found that they carried one or other of two allelomorphs, say $\mathbf{a}_1$ and $\mathbf{a}_2$. He then isolated twenty-five single spores from a second fruit-body, obtained from a different locality, and found that they carried one or other of two different allelomorphs, say $\mathbf{a}_3$ and $\mathbf{a}_4$, with the exception of one spore which carried the allelomorph $\mathbf{a}_1$. Vandendries concluded that this proved that a mutation had occurred from either $\mathbf{a}_3$ or $\mathbf{a}_4$ to $\mathbf{a}_1$. He overlooked the fact that the chance of a mutation happening to take place to either of the two allelomorphs carried by the other fruit-body, is so remote that grave suspicion must rest on his claim for mutation. It seems much more likely that a spore from the first fruit-body strayed into one of the cultures. Later, Vandendries (1925*c*) claimed that mutation had occurred in many of his cultures of *C. radians*, since a large proportion of them developed clamp-connexions. However, he made no attempt to prove this claim by investigating the identity of the new allelomorphs. Similarly, with *C. micaceus*, Vandendries (1926, 1927*a–c*, 1928, 1929*a*, *b*) purported to find many examples of mutation. However, in no case were the allelomorphs of the parent cultures nor those of the 'mutants', identified. Supposed cases of mutation to 'sterility' (Vandendries, 1929*b*,

p. 227) can only mean that a mutation had occurred from one allelomorph to the allelomorph specifically present in the single-spore culture with which it was paired, which by the laws of probability is an exceedingly unlikely occurrence. Vandendries admits that some of his cultures occasionally became infected with other fungi: the possibility that some, at least, of the irregularities of behaviour which he attributes to mutation may, in fact, have been due to infection by spores or oidia of *C. micaceus* containing nuclei with different allelomorphs, should not therefore be overlooked.

Hanna (1925) claimed to have found mutations at the loci for heterothallism in *C. lagopus*, but although he isolated and paired together ten single-spore cultures from fruit-bodies (called *F* and *G*), formed by a single-spore culture which spontaneously developed clamp-connexions, he did not attempt to identify the allelomorphs present, by pairing them with his original single-spore cultures. Eight of the ten single spores isolated from fruit-bodies *F* and *G* belonged to four complementary mating types (e.g. $\mathbf{a}_1\,\mathbf{b}_1$, $\mathbf{a}_2\,\mathbf{b}_2$, $\mathbf{a}_1\,\mathbf{b}_2$ and $\mathbf{a}_2\,\mathbf{b}_1$), but the other two (called F_1 and F_3) were alike and carried at both loci allelomorphs that were different from all the rest (e.g. $\mathbf{a}_3\,\mathbf{b}_3$). This suggests that spores F_1 and F_3 were stray spores from another fruit-body and not mutations.

Newton (1926*a*) found that many single-spore cultures of *C. rostrupianus* spontaneously developed clamp-connexions in the course of 6 months. She discussed the possibility that this was due to mutation, but made no tests.

Brunswik (1926) isolated the four spores from each of ninety-three basidia of *C. fimetarius*, and in four of the tetrads obtained three spores with one allelomorph and one with another, at one or other of the two loci, instead of two spores with one allelomorph and two with the other. He attributed this to mutation from one allelomorph to the other, but it seems much more likely to have been due to stray spores from other basidia.

As evidence against the frequent occurrence of mutations, Hanna (1928) reported that no clamp-connexions had formed spontaneously in any of his cultures of *C. lagopus* during a period of 3 years. This is perhaps not surprising since *C. lagopus* is tetrapolar and mutation at both loci would be necessary before clamp-formation could occur. However, Mounce & Macrae (1938) reported that they kept numerous single-spore cultures of the bipolar species *Fomes pinicola* for 10 years without any mutations having taken place. Fries (1940) found that single-spore cultures of *Cyathus striatus* remained unchanged in mating type for 2½ years, and the small number of allelomorphs present in the populations of *C. striatus* and *Crucibulum vulgare* (Fries, 1943) suggests that mutation to new allelomorphs is of rare occurrence. Furthermore, the very widespread occurrence in the Hymenomycetes and Gasteromycetes of only two allelomorphs per locus amongst the spores from individual wild fruit-bodies implies a relatively low mutation rate.

There seems good reason, therefore, for believing that, contrary to the commonly held opinion, mutation from one allelomorph to another at the loci for heterothallism in the Hymenomycetes and Gasteromycetes is not in general of frequent occurrence.

6. THE DISTRIBUTION OF THE ALLELOMORPHS AT THE LOCI FOR HETEROTHALLISM IN NATURAL POPULATIONS OF SPECIES

The misleading term 'geographic races' was applied by Brunswik (1924) and Kniep (1928) to individuals of species of Hymenomycetes from different localities which carried different allelomorphs, despite the fact that Kniep (1922) had demonstrated that such individuals merely differed in the allelomorphs present at the loci for heterothallism. The term 'geographic race' has since been widely used to describe full cross-fertility between the single-spore cultures of two different fruit-bodies of a species. With the discovery of true geographic races in *Coprinus micaceus*, showing intersterility in all or most pairings, Vandendries (1929*a*) condemned Brunswik and Kniep's use of the term. The false notion that multiple allelomorphs are normally distributed through the population of a fungus species on a geographical basis appears to have arisen through the impossibility of defining in precise terms the limits of an individual in nature. It was observed that two fruit-bodies of the same species collected in proximity usually carried the same allelomorphs, whereas fruit-bodies from far apart rarely did so, and it was therefore assumed that each pair of allelomorphs was characteristic of a certain locality. It is now evident that the 'locality' for a particular pair of allelomorphs is defined merely by the limits of the vegetative spread of the mycelium of one fungus individual. Fruit-bodies collected from a limited area may, in general, be expected to carry as many different allelomorphs as those collected from widely separated localities, so long as only one fruit-body is sampled from each fungus individual. Exception must be made of fungi such as the Nidulariales in which the dispersal of allelomorphs through the population appears to be much restricted, probably owing to the retention of the basidiospores within a peridiolum. However, in the majority of the Hymenomycetes and Gasteromycetes, the very large number of basidiospores produced by each fruit-body and the efficient spore dispersal by wind must in general result in allelomorphs having a very wide distribution. Evidence in support of this generalization is presented later in this section.

That two fruit-bodies growing in close proximity in nature do not necessarily carry the same allelomorphs was first shown by Vandendries (1923*b*), who found complete fertility in all pairings of single-spore cultures of two fruit-bodies of *Panaeolus campanulatus* which were growing 3 m. apart. Likewise, Hanna (1925) found that three fruit-bodies of *Coprinus lagopus* from the same stable, including two from the same sample of dung, carried different pairs of allelomorphs at both loci. Barnett (1937) obtained complete fertility between single-spore cultures of two fruit-bodies of *Exidia glandulosa* growing only 2 in. apart on the same log, and in *E. saccharina*, he found two fruit-bodies from a stick 12 in. long carried a different pair of allelomorphs. In their study of pairing reactions of single-spore cultures of *Fomes pinicola*, Mounce & Macrae (1938) found that two fruit-bodies from the same tree carried the same pair of allelomorphs in four instances, but in two others they carried a different pair of allelomorphs. These examples show that many allelomorphs may be present in the mycelium of a species even in quite a limited area.

The widespread distribution of individual allelomorphs was first demonstrated by Brunswik (1924) in *Coprinus comatus*. He found the same allelomorph in three fruit-bodies, one from Vienna, one from St Pölten (Austria), and one from Hamburg. Mounce & Macrae (1937) found the same allelomorph in two fruit-bodies of *Fomes roseus*, one from Gaspé County, Quebec, and one from Keewatin, Ontario. Fries & Jonasson (1941) found that a sample of six fruit-bodies of the tetrapolar species *Polyporus abietinus* from near Uppsala had three allelomorphs in common with a sample of eight fruit-bodies collected 380 km. away near Uddevala in western Sweden. In the sample of fruit-bodies from Uppsala, collected from sources several kilometres apart, three allelomorphs appeared more than once, and one allelomorph was recorded more than once in the sample from Uddevala. Thus within each sample from a limited area of less than 5 km. radius there were no more allelomorphs in common than between the two samples from widely separated areas. This suggests that the allelomorphs for heterothallism in *P. abietinus* are not restricted on a geographical basis in Sweden.

In the Nidulariales, the distribution of allelomorphs within the population of a species appears to be on quite a different basis from that found in the Hymenomycetes. Fries (1940) investigated nine fruit-bodies of the tetrapolar species *Cyathus striatus*. He found that four of them carried the same pair of allelomorphs at both loci; three of them were from localities within 80 km. of one another in eastern Sweden, but the fourth was from Latvia, 700 km. away. Of the other five fruit-bodies, three were from Sweden, one from Holland and one from Switzerland, and all were found to carry at least two allelomorphs (considering the two loci together) that were also possessed by several others of the sample. In the tetrapolar species *Crucibulum vulgare*, Fries (1943) compared nine fruit-bodies from the neighbourhood of Uppsala with six from near Uddevala, 380 km. away. In many cases, fruit-bodies from localities within 1 or 2 km. of one another were found to carry identical allelomorphs at both loci. At the *a* locus only three allelomorphs were found altogether, and all three were present in both the Uppsala and Uddevala populations. At the *b* locus, five allelomorphs were found in the Uppsala population and six in that from Uddevala, with none in common. From this work it is evident that the allelomorphs for heterothallism in *Cyathus striatus* and *Crucibulum vulgare*, besides being fewer in number than in the Hymenomycetes, show a different distribution within the population of the species. First, there is a tendency for fruit-bodies growing within a few kilometres of one another to have identical allelomorphs. This implies that a number of basidiospores, probably enclosed in a peridiolum, are frequently dispersed together. Secondly, there is a tendency for the populations of particular regions to have characteristic allelomorphs distinct from those of populations some distance away as, for instance, at the *b* locus of *C. vulgare*. This is perhaps due to dispersal of spores by animals instead of wind, which would have the effect of reducing the spread of allelomorphs through the population, particularly if topographical features restricted the movement of the animals concerned.

In the Hymenomycetes and Gasteromycetes, as in all sexually reproducing organisms, isolating mechanisms may occur which restrict the spread of allelomorphs through the population of a species. Vandendries (1927*a–c*) and Vandendries & Robyn (1929) found several partially intersterile races of *Coprinus micaceus* in Europe and America, but the number and distribution of the races was rather obscure. Barnett (1937) found considerable sterility between single-spore cultures of *Auricularia auricula-judae* obtained from fruit-bodies that were growing on coniferous trees, and those from fruit-bodies on non-coniferous trees. Verrall (1937) found three intersterile subspecies of *Fomes igniarius*, which were correlated with different host species. Mounce & Macrae (1938) found two largely intersterile races of *F. pinicola* which occurred in similar habitats in North America and had similar geographical distributions. In these examples, various isolating mechanisms appear to be involved as the primary factors in the division of the species into intersterile races: in *Coprinus micaceus*, geographical isolation by long distances would appear to be the main factor; in *Auricularia auricula-judae* and *Fomes igniarius* a physiological or genetic isolating mechanism appears to have initiated the formation of races adapted to particular host species; and in *F. pinicola* the isolating mechanism appears to be purely genetic, not associated with geographical or ecological factors. Such partially or wholly intersterile races or subspecies will of course act as barriers to the spread of allelomorphs for heterothallism through the whole population. Any new allelomorphs arising by mutation are likely to be confined to the race in which they appear.

7. THE NUMBER OF ALLELOMORPHS AT THE LOCI FOR HETEROTHALLISM IN NATURAL POPULATIONS OF SPECIES

The numbers of allelomorphs at the loci for heterothallism that have been recorded in various species of Hymenomycetes and Gasteromycetes, are given in Table 1. In addition to the data listed in the table, Kniep (1928) reported that he obtained fifteen fruit-bodies of *Schizophyllum commune*, the spores of which were all mutually interfertile, implying the presence of thirty allelomorphs at each locus, but he did not publish his results in detail. A number of other species have been investigated without the identification of individual allelomorphs. Vandendries (1927*a–c*) and Vandendries & Robyn (1929) made a very extensive survey of the pairing reactions of spores from fruit-bodies of *Coprinus micaceus* from different sources, but individual allelomorphs were not identified, and therefore the number of them in the sample cannot be estimated. Vandendries (1930*a*) investigated the pairing reactions of the spores from twelve fruit-bodies of *Psathyrella disseminata*, but gave no details other than to state that almost all showed complete cross-fertility. Mounce & Macrae (1938) studied the pairing reactions of the spores from over fifty fruit-bodies of *Fomes pinicola* from different sources, but their investigation was likewise conducted without identifying individual allelomorphs. Similarly, Macrae (1942) analysed the pairing reactions of the spores from a number of fruit-bodies of *Panus stypticus*, and Nobles (1943) investigated the interfertility of the spores of different fruit-bodies of *Trametes serialis*, *Poria microspora* and *Polyporus palustris*, but in none of these instances can the number of allelomorphs in the samples studied be estimated.

Table 1. *The number of allelomorphs at the loci for heterothallism that have been recorded in various species of Hymenomycetes and Gasteromycetes*

Author	Species	Number of fruit-bodies analysed from different sources	Maximum number of allelomorphs possible in sample		Number of allelomorphs recorded	
(*a*) Bipolar Hymenomycetes						
Brunswik, 1924	*Coprinus comatus*	5½	11		9	
Mounce & Macrae, 1937	*Fomes roseus*	5	10		9	
Mounce & Macrae, 1937	*F. subroseus*	10	20		20	
Barnett, 1937	*Auricularia auricula-judae*	5	10		10	
(*b*) Tetrapolar Hymenomycetes						
			a	*b*	*a*	*b*
Brunswik, 1924	*Coprinus fimetarius* (L.) Fr.	13½	27	27	27	27
Hanna, 1925	*C. lagopus* Fr. sensu Buller = *C. fimetarius* (L.) Fr.	7	14	14	14	14
Fries & Jonasson, 1941	*Polyporus abietinus*	14	28	28	23	26
(*c*) Tetrapolar Gasteromycetes						
Fries, 1940	*Cyathus striatus*	9	18	18	4	5
Fries, 1943	*Crucibulum vulgare*	15	30	30	3	11

The data presented in Table 1, together with the less precise cross-fertility studies discussed above, show clearly that a large number of allelomorphs are present at the loci for heterothallism in all the species of Hymenomycetes that have been studied. Thus

Brunswik (1924) recorded twenty-seven allelomorphs at each of the two loci in *Coprinus fimetarius*, and Mounce & Macrae (1937) recorded twenty allelomorphs in the bipolar species *Fomes subroseus*. In neither of these investigations did an allelomorph occur more than once in the sample of fruit-bodies examined. This means that the total number of allelomorphs in the populations of these two species must be very much greater than the number actually recorded. In *Polyporus abietinus*, Fries & Jonasson (1941) found a total of forty-nine allelomorphs at the two loci, out of fifty-six (twenty-eight at each locus) possible in the sample of fruit-bodies studied. This again implies a much larger figure than the recorded number, for the total number of allelomorphs in the population, though not as many as in the population of *Coprinus fimetarius* studied by Brunswik.

The number of allelomorphs present in natural populations of two species of Gasteromycetes belonging to the order Nidulariales has been studied by Fries (1940, 1943). As already indicated in the last section, he found that the number of allelomorphs was small compared with the number recorded in the Hymenomycetes. In *Cyathus striatus* he found a total of only nine allelomorphs at the two loci out of a possible thirty-six in the sample studied, and in *Crucibulum vulgare* only fourteen out of a possible sixty. The fruit-bodies were selected from a wide area, so there was no question of including more than one fruit-body from one individual. There can be little doubt, as Fries has pointed out, that the small number of allelomorphs in the populations of these two species of Nidulariales is associated with the fact that the unit of dispersal in this group of fungi is the peridiolum and not the basidiospore. Each peridiolum contains the spores from a considerable number of basidia, and so there is probably a high degree of inbreeding in these species. It is also possible that dispersal of the peridiola takes place by animals, which might also lead to closer inbreeding than would occur with wind dispersal.

It has been shown in section 6 that, apart from special instances such as species of Nidulariales, it is probable that the allelomorphs at the loci for heterothallism are distributed at random through the population of a species, except where geographical or other barriers restrict their spread. Furthermore, as discussed in section 5, the mutation rate from one allelomorph to another appears, in general, to be low. Hence, within an interbreeding group of individuals in nature through which the allelomorphs are distributed at random, the majority of the allelomorphs are likely to occur with approximately equal frequencies, since all are presumably subject to similar conditions as regards natural selection. A rough estimate of the number of allelomorphs in natural populations of Hymenomycetes and Gasteromycetes can therefore be made on the assumption that all the allelomorphs are equally frequent in the population. If the sample of fruit-bodies analysed is a random one from the population as a whole, then an estimate of the total number of allelomorphs can be obtained from the frequency with which allelomorphs occur more than once in the sample studied.

If at one locus there are N allelomorphs in the whole population, of which n have been recorded in a sample consisting of r fruit-bodies (pairs of allelomorphs), then the chance that one additional fruit-body will contain an allelomorph not previously recorded is $2 \times \frac{N-n}{N}$. In other words, if δn represents a small increase in n associated with an increase δr in r, then $\frac{\delta n}{\delta r} = 2 \times \frac{N-n}{N}$. In the limiting case, as $\delta n \to 0$, $\frac{dn}{dr} = 2 \times \frac{N-n}{N}$.

Therefore
$$r=\int \frac{N}{2\,(N-n)}\,dn$$
$$=-\frac{N}{2}\log\,(N-n)+c,$$

where c is a constant. As $n=0$ when $r=0$, $c=\frac{N}{2}\log N$, therefore

$$r=\frac{N}{2}\log\left(\frac{N}{N-n}\right),$$

therefore
$$2r=N\log\left(\frac{1}{1-n/N}\right),$$

therefore
$$\frac{n}{2r}=\frac{n}{N}\,\frac{1}{\log\left(\frac{1}{1-n/N}\right)}.$$

From this expression, values of $n/2r$, the proportion of different allelomorphs present in the sample, can be calculated for particular values of n/N, the proportion of the total number of allelomorphs in the population that have been recorded. In Fig. 1, $n/2r$ is plotted against n/N, and from this graph values of n/N and hence of N can be obtained for given values of $n/2r$.

This method for obtaining a rough estimate of the number of allelomorphs in populations of species of Hymenomycetes and Gasteromycetes has been applied to the data in Table 1. In the instances in which no allelomorph has been recorded more than once, the logical deduction would be that the number of allelomorphs in the population is infinite. In reality this merely reflects the inadequate size of the sample studied. Brunswik's data for *Coprinus comatus* in Central Europe, and Mounce & Macrae's for *Fomes roseus* in Canada, lead to estimates of 30 and 50, respectively, for the total number of allelomorphs in the populations studied. Fries's data for *Polyporus abietinus* in Sweden give estimates of 70 and 200 allelomorphs at the *a* and *b* loci, respectively. It is concluded that the number of allelomorphs at the loci for heterothallism in natural populations of species of Hymenomycetes and Gasteromycetes is generally of the order of magnitude of 100 per locus.

In the order Nidulariales of the Gasteromycetes, presumably owing to restricted spore dispersal, the allelomorphs do not appear to be distributed at random through the population of a species in one geographical region. Consequently, it is only possible to estimate the total number of allelomorphs in the population of a limited area, in which the individuals may be considered to represent a natural interbreeding group, and in which the allelomorphs may be expected to be present with approximately equal frequencies. In Fries's investigation of *Crucibulum vulgare*, not more than six allelomorphs were recorded at one locus in two samples of six and nine fruit-bodies respectively from two limited areas of a few square kilometres in Sweden. This implies that the total number of allelomorphs present in the populations of these local breeding units is probably of the order of magnitude of 10 per locus. Fries's data for *Cyathus striatus* suggest that in the whole of North-western Europe there may not be more than approximately ten allelomorphs at each locus in this species. It is thus evident that whereas the number of allelomorphs in natural populations of species with efficient spore dispersal, is probably of the order of magnitude of 100 per locus, in the Nidulariales, where spore dispersal would appear to be much less efficient, the number of allelomorphs appears to be reduced, presumably owing to inbreeding, to the order of magnitude of 10 per locus.

The method suggested by Bateman (1947) for estimating the number of allelomorphs at the incompatibility locus in populations of flowering plants can be applied to fungi with multiple-allelomorph heterothallism, and gives results similar to those obtained by the method described above.

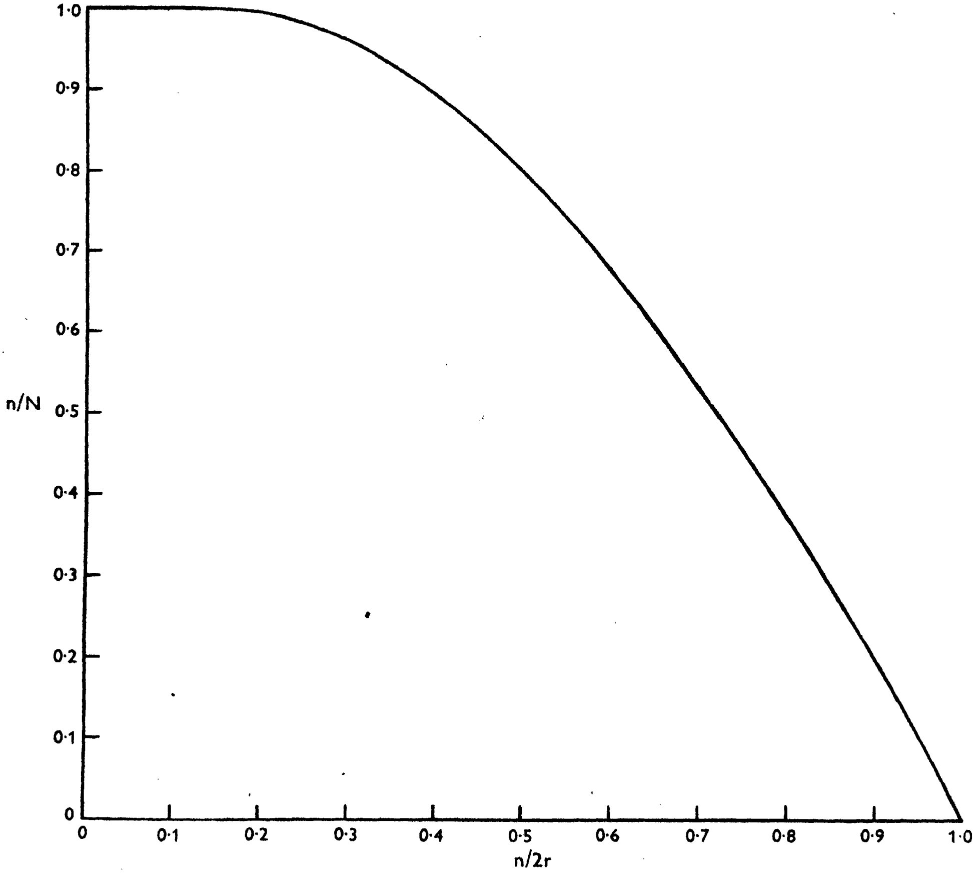

Fig. 1. Graph to show the number of allelomorphs, N, in a natural population in which all the allelomorphs are assumed to be equally frequent, and from which r fruit-bodies have been sampled and found to contain n different allelomorphs. The proportion of different allelomorphs, $n/(2r)$, present in the sample is plotted against the proportion of the total number of allelomorphs in the population that have been recorded, n/N.

8. LOCATION OF THE TWO LOCI FOR HETEROTHALLISM IN TETRAPOLAR SPECIES

(a) *Linkage data*

The data from which it is possible to test for linkage between the two loci for heterothallism in tetrapolar species, are meagre. In much of the breeding work insufficient details have been published to make a satisfactory linkage test possible: in many instances the genetic constitution of the parents of a particular cross is not given so that it is not known which of the two pairs of classes of individuals in the progeny ($\mathbf{a}_1\,\mathbf{b}_1+\mathbf{a}_2\,\mathbf{b}_2$ and $\mathbf{a}_1\,\mathbf{b}_2+\mathbf{a}_2\,\mathbf{b}_1$) constitutes the parental combinations of the genes and which the recombinations. In the analysis of the spores of a wild fruit-body the parental and recombinant classes can of course never be identified. If there is a significant difference in the size of the two classes, then linkage is suspected, but it cannot be proven without knowledge of the constitution of the parents.

Data from breeding experiments in which the parentage is known, are given in Table 2. Kniep's results for *Schizophyllum commune* give forty-six parental combinations and forty-five recombinations. There is thus no evidence for linkage of the two loci for heterothallism in *S. commune*. The data of Brunswik for *Coprinus fimetarius* comprise eighty-seven parental combinations and ninety-eight recombinations of the allelomorphs and so in this species also there is no evidence for linkage. In *Galera tenera* Oikawa's extensive data total 164 parental combinations and 140 recombinations. These figures are not significantly different from equality, and the figure of 46% recombination which they give may easily be a chance deviation from 50%. Macrae (1942, p. 426) gives some results for *Panus stypticus*. In a breeding experiment she obtained twelve parental combinations and eight recombinations of the pairs of allelomorphs. She also gives eight sets of data from wild fruit-bodies, as follows: (6, 3), (18, 3), (11, 17), (6, 3), (7, 2), (6, 4), (8, 19), (6, 7). The pairs of figures within brackets are the numbers of spores in the two pairs of classes ($\mathbf{a}_1\,\mathbf{b}_1+\mathbf{a}_2\,\mathbf{b}_2$ and $\mathbf{a}_1\,\mathbf{b}_2+\mathbf{a}_2\,\mathbf{b}_1$) for each fruit-body analysed. There is a suggestion of linkage in some of the data, but in the absence of knowledge of which are parental and which recombinant classes, no definite conclusion can be reached.

Table 2. *Linkage data for the two loci for heterothallism in certain tetrapolar species of Hymenomycetes*

Species	Reference	Parental combinations	Recombinations
Schizophyllum commune	Kniep:		
	(1922, p. 19)	10	10
		14	10
	(1923, p. 175, table 3)	9	13
	(1923, p. 177, table 4)	13	12
	Total	46	45
Coprinus fimetarius	Brunswik:		
	(1924, p. 58)	20	18
		16	20
	(1924, p. 59)	9	9
	(1924, p. 62)	15	12
	(1924, p. 75)	11	11
	(1924, p. 76)	8	13
	(1924, p. 77)	8	15
	Total	87	98
Galera tenera	Oikawa:		
	(1939, pp. 249–51, table 2)	95	85
	(1939, pp. 254–5, table 5)	69	55
	Total	164	140

In section 9 of this paper, the numbers of spores in the two pairs of classes ($\mathbf{a}_1\,\mathbf{b}_1+\mathbf{a}_2\,\mathbf{b}_2$ and $\mathbf{a}_1\,\mathbf{b}_2+\mathbf{a}_2\,\mathbf{b}_1$) are given for sixty-four other tetrapolar species on the basis of analyses that have been made with wild fruit-bodies, in which the parental combinations are necessarily unknown. In a majority of these there is no evidence for linkage, but in the following cases there is a significant deviation (5% probability) from equality in the frequencies of the two classes:

Polystictus zonatus	(21, 7)	(Vandendries & Brodie, 1933*c*)
Collybia tuberosa	(14, 5)	(Harnack, 1931); (14, 3) (Arnold, 1935)
Coprinus auricomus	(12, 3)	(Quintanilha, A. and L. & Vasermanis, 1941)
Pleurotus pinsitus	(10, 2)	(Vandendries, 1934*b*)

The figures in brackets are the frequencies recorded for the two pairs of classes of spores. Linkage between the two loci for heterothallism is suspected in these species, but breeding experiments are required in order to establish its existence with certainty. There is also a suggestion of linkage in Quintanilha's (1944) data of (13, 7), (14, 7) and (8, 13) for *Hypholoma prona*.

Mather (1942) has pointed out that the occurrence of two loci for heterothallism promotes outbreeding by decreasing the frequency of fertile combinations among the spores from one fruit-body. The maximum efficiency of outbreeding is found when the loci are not linked. It is not surprising, therefore, to find that out of sixty-eight tetrapolar species for which data are available, there are only six in which there is a suggestion of linkage between the loci. However, it must be admitted that most of the data are inadequate to detect slight linkage.

(*b*) *Data from the analysis of tetrads*

Kniep (1922) was the first to investigate the pairing reactions of the individual spores of a single basidium. In *Aleurodiscus polygonius* he, at first, found spores of only two mating types on each basidium, for example, two $\mathbf{a}_1\,\mathbf{b}_1$ and two $\mathbf{a}_2\,\mathbf{b}_2$ spores, or two $\mathbf{a}_1\,\mathbf{b}_2$ and two $\mathbf{a}_2\,\mathbf{b}_1$. Funke (1924) and Hanna (1925) independently discovered that individual basidia in tetrapolar species may have four spores all of different mating types, that is, one $\mathbf{a}_1\,\mathbf{b}_1$, one $\mathbf{a}_2\,\mathbf{b}_2$, one $\mathbf{a}_1\,\mathbf{b}_2$ and one $\mathbf{a}_2\,\mathbf{b}_1$. Dodge (1940) was the first to point out that these four-mating-type basidia can be explained by crossing-over, but he did not determine the precise significance of the relative frequencies of two- and four-mating-type tetrads of spores.

If the frequencies of second-division segregation at the a and b loci are x and y respectively, then the corresponding frequencies of first-division segregation are $1-x$ and $1-y$. It is assumed that there is no linkage between the a and b loci. First-division segregation at both loci will occur with frequency $(1-x)(1-y)$ and will give two-mating-type tetrads only. First-division segregation at one locus and second-division segregation at the other will occur with frequency

$$x(1-y)+y(1-x)=x+y-2xy.$$

This will give rise to four-mating-type tetrads only. Second-division segregation at both loci will occur with frequency xy, and will give two- and four-mating-type tetrads with equal frequency. Hence the frequency of four-mating-type tetrads is given by the expression

$$x+y-2xy+\frac{xy}{2}=x+y-\frac{3xy}{2}.$$

In Fig. 2 are shown lines of constant frequency of four-mating-type tetrads for various frequencies of second-division segregation at the two loci. It will be observed that: (1) frequencies of four-mating-type tetrads below 50% imply that the frequencies of second-division segregation at both loci are under 50%; (2) frequencies of four-mating-type tetrads between 50 and 66·7% imply that the frequencies of second-division segregation at the two loci are either both under or both over 66·7%; and (3) frequencies of four-mating-type tetrads above 66·7% imply that the frequencies of second-division segregation at the two loci are respectively below and above 66·7%. Small frequencies of four-mating-type tetrads are approximately equal to the sum of the respective frequencies of second-division segregation for the two loci; larger frequencies are appreciably less than the sum; and frequencies of four-mating-type tetrads greater than 66·7% approach the mean of the two second-division segregation frequencies. When the frequency of four-mating-type tetrads is 66·7%, the frequency of second-division segregation at one locus must also be 66·7%, but the frequency at the other locus is quite indeterminate. Since frequencies of second-division segregation much in excess of 66·7% are likely to be rare (Mather, 1938), frequencies of four-mating-type tetrads above this figure are also likely to be uncommon.

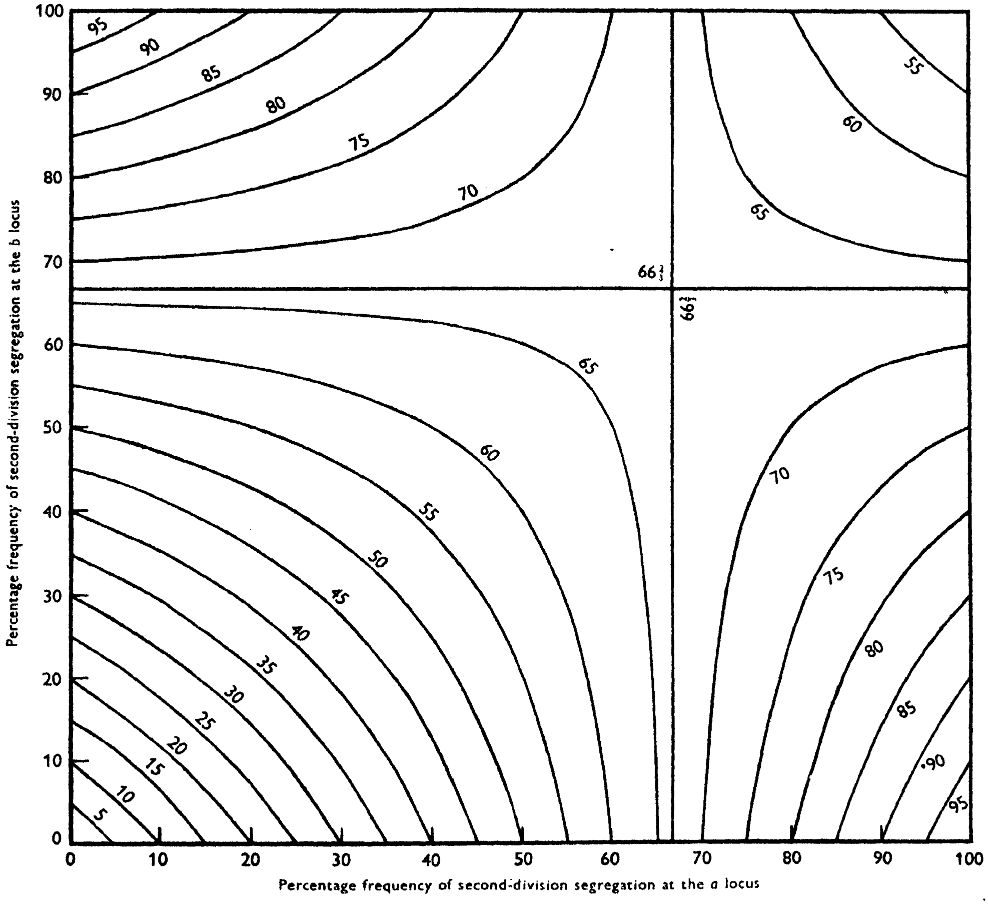

Fig. 2. Lines of constant frequency of four-mating-type tetrads are drawn for various percentage frequencies of second-division segregation at the two loci in tetrapolar species. The figure alongside each line is the percentage frequency of four-mating-type tetrads.

Published data for the relative frequencies of two- and four-mating-type tetrads in tetrapolar species of Hymenomycetes are summarized in Table 3. In very few cases is it known which of the two kinds of two-mating-type tetrads represents the parental combinations of the allelomorphs and which the recombinations. The two figures with a plus sign between them in the column headed two-mating-type tetrads give these frequencies, where known, without attempting to distinguish the parental combinations and the recombinations.

Table 3. *The frequencies of two- and four-mating-type tetrads recorded in various tetrapolar species of Hymenomycetes*

Species	Author	Source	Frequency of tetrads			
			Two mating types	Four mating types	Total	Four mating types (%)
Aleurodiscus polygonius	Kniep (1922)	Germany:				
		Nov. 1920	35 } 64	0 } 6	35 } 70	0 } 8·6
	Kniep (1928, p. 406)	Sept. 1921	18	5	23	21·7
		Feb. 1924	11	1	12	8·3
Hypholoma fasciculare	Funke (1924)	Germany	2	4	6	66·7
Collybia velutipes	Funke (1924)	Germany	1	3	4	75·0
Coprinus lagopus Fr. sensu Buller = *C. fimetarius* (L.) Fr.	Hanna (1925)	Canada	7 } 29	6 } 31	13 } 60	46·1 } 51·6
	Newton (1926)	Canada	[9 + 8] 22	25	47	53·2
C. fimetarius (L.) Fr.	Brunswik (1926)	Austria	29 + 27 = 56	37	93	39·8
	Oort (1930)	Holland	77	74	151	49·0
	Bohn (1934)	Germany:				
		Fruit-body A	1 + 1 = 2 } 51	2 } 46	4 } 97	50·0 } 47·4
		Fruit-body B	0 + 1 = 1	3	4	75·0
		Fruit-body C	3 + 0 = 3	2	5	40·0
		Fruit-body D	4 + 3 = 7	3	10	30·0
		Fruit-body E	23 + 15 = 38	36	74	48·6
	Quintanilha (1933)	Portugal:				
		Wild fruit-body	1 + 5 = 6 } 66	11 } 139	17 } 205	64·7 } 67·7
		Fruit-body in culture	11 + 10 = 21	86	107	80·4
	Quintanilha (1935, p. 311)		2 + 3 = 5	7	12	58·3
	Quintanilha (1937)		8 + 4 = 12	16	28	57·2
			13 + 9 = 22	19	41	46·3
	Totals for *Coprinus fimetarius*		279	327	606	53·9

In *Aleurodiscus polygonius*, the mean frequency of four-mating-type tetrads, on the basis of the analysis of seventy basidia by Kniep, is 8·6%. This means that at neither locus can there have been more than 8·6% second-division segregation. Hence both loci must be within 4·3 units of the centromeres of their respective chromosomes. If they were located on the same chromosome they would be closely linked, with not more than 4·5% of recombination (see Fig. 2), but from the data given in section 9, it is quite clear that the loci are not linked. It is concluded that the two loci for heterothallism in *Aleurodiscus polygonius* are situated near to the centromeres of different chromosomes.

The only other species for which extensive data exist is *Coprinus fimetarius*. Data for a total of 606 tetrads are given in Table 3, of which 327, or 53·9% were four-mating type. The data, however, are not all consistent. The ninety-three tetrads analysed by Brunswik (1926) gave a significantly lower figure of 39·8%, and the 107 tetrads investigated by

Quintanilha (1933) gave a significantly higher figure of 80·4%. It would appear that the frequency of recombination in these two investigations was significantly different from that in the remainder. Quintanilha's later analyses of tetrads of *C. fimetarius* show a much lower frequency of four-mating-type tetrads, and are consistent with the data of Hanna, Newton, Oort and Bohn.

From the mean figure of 53·9% for the frequency of four-mating-type tetrads in *C. fimetarius*, the following conclusions can be drawn regarding the location of the two loci. If both loci had the same frequency of second-division segregation, the value of this frequency would have to be either 37·5 or 95·9%, in order to give 53·9% of four-mating-type tetrads. It is unlikely that the two second-division-segregation frequencies are in fact equal. By reference to Fig. 2 it can be seen that the frequencies of second-division segregation must be either less than 37·5% at one locus and between 37·5 and 53·9% at the other, or between 92·2 and 95·9% at one locus and over 95·9% at the other. The second alternative, with over 90% second-division segregation at both loci, is most unlikely to be the true one (see above), and so it would appear that the loci are, respectively, less than 18·7 units and between 18·7 and 27·0 units from the centromeres of their respective chromosomes (all distances are necessarily uncorrected for double cross-overs). There is no evidence for linkage between the loci and so these distances probably refer to the centromeres of different chromosomes.

9. LIST OF HOMOTHALLIC AND OF BIPOLAR AND TETRAPOLAR HETEROTHALLIC SPECIES OF HYMENOMYCETES AND GASTEROMYCETES

In the following list the terms bipolar and tetrapolar imply the existence of one and two loci for heterothallism respectively; the word heterothallic is used only when the number of loci is unknown. No reference to multiple allelomorphs implies that no cross-fertility tests have been carried out. With the tetrapolar species, the pairs of figures in brackets after the word tetrapolar give the number of spores that were recorded in classes $\mathbf{a}_1\,\mathbf{b}_1+\mathbf{a}_2\,\mathbf{b}_2$ and in $\mathbf{a}_1\,\mathbf{b}_2+\mathbf{a}_2\,\mathbf{b}_1$ respectively (formulae arbitrary). If more than one fruit-body has been analysed, separate pairs of figures are given for each, since in no case is it known which figure represents the parental combinations of the allelomorphs and which the recombinations. A significant deviation from equality in the pairs of figures implies that the *a* and *b* loci may be linked.

HYMENOMYCETES

Tremellales

AURICULARIACEAE

Auricularia auricula-judae (L. ex Fr.) Schroet. Bipolar, multiple alleles (Barnett, 1937).
Phleogena faginea (Fr.) Link. Homothallic (Shear & Dodge, 1925).
Pilacre faginea (Fr.) B. & Br. *See* Phleogena faginea (Fr.) Link.

TREMELLACEAE

Exidia glandulosa (Bull.) Fr. Bipolar, multiple alleles (Barnett, 1937).
E. nucleata (Schw.) Burt. Bipolar (Barnett, 1937).
E. recisa (Ditm.) Fr. Bipolar, multiple alleles (Barnett, 1937).
E. saccharina Fr. Bipolar, multiple alleles (Barnett, 1937).
Sebacina calcea (Pers.) Bres. Heterothallic (Kniep, 1922).

DACRYMYCETACEAE

Calocera cornea Batsch. Secondarily homothallic (Vandendries, 1937*b*, *c*).

Agaricales

EXOBASIDIACEAE

None investigated.

THELEPHORACEAE

Aleurodiscus canadensis Skolko. Bipolar, secondarily homothallic (Skolko, 1944).
A. polygonius (Pers.) v. Höhn & Litsch. Tetrapolar (104, 106) (47, 39) (Kniep, 1922).
Corticium calceum Burt. Heterothallic (Biggs, 1938).
C. centrifugum (Lev.) Bres. Tetrapolar (15, 15), multiple alleles (Butler, 1930).
C. conigenum. Bipolar (Shear & Davidson, 1944).
C. coronilla v. Höhn & Litsch., group I. Bipolar, multiple alleles (Biggs, 1937).
C. coronilla v. Höhn & Litsch., group II. Homothallic (Biggs, 1937).
C. coronilla v. Höhn & Litsch., group III. Tetrapolar (9, 11), multiple alleles (Biggs, 1937).
C. coronilla v. Höhn & Litsch., group IV. Bipolar (Biggs, 1937).
C. effuscatum Cooke & Ellis. Tetrapolar (13, 11), multiple alleles (Nobles, 1942).
C. hydnans (Schw.) Burt. Heterothallic (Biggs, 1938).
C. incrustans v. Höhn & Litsch. Bipolar (Nobles, 1937).
C. laeve Pers. Heterothallic (Biggs, 1938).
C. polygonium Pers. Heterothallic (Biggs, 1938).
C. porosum (B. & G.) Wakef. Heterothallic (Biggs, 1938).
C. praticola Kotila. Homothallic (Kotila, 1929).
C. radiosum Fr. Heterothallic (Biggs, 1938).
C. serum Fr. Tetrapolar (Kniep, 1920, 1928; Lehfeldt, 1923).
C. stramineum Bres. Heterothallic (Biggs, 1938).
C. vagum (Berk. & Curt.) (syn. Hypochnus Solani Prill. & Del.). Homothallic (Müller, 1924).
C. varians Kniep. Heterothallic (Kniep, 1922).
Cytidia salicina (Fr.) Burt. Tetrapolar (14, 11) (Biggs, 1938).
Gloeocystidium roseo-cremeum Kuhn. *See* Peniphora roseo-cremeum (Bres.)
Hypochnus solani Prill. & Del. *See* Corticium vagum (Berk. & Curt.).
H. terrestris Kniep. *See* Tomentella terrestris (Kniep).
Peniophora affinis Burt. Bipolar (Biggs, 1938).
P. allescheri Bres. Bipolar (Nobles, 1935).
P. candida (Pers.) Lyman. Tetrapolar (Biggs, 1938).
P. ciliata (Fr.) Bres. Heterothallic (Kniep, 1922).
P. cinerea (Pers.) Cooke. Tetrapolar (Vandendries, 1937*b*; Biggs, 1938).
P. corticalis (Bull.) Bres. Heterothallic (Kniep, 1920).
P. farinosa Bres. Tetrapolar (Biggs, 1938).
P. incarnata (Pers.) Karst. Tetrapolar (Biggs, 1938).
P. laevis (Fr.) Burt. Heterothallic (Biggs, 1938).
P. ludoviciana Burt. Bipolar (Biggs, 1938).
P. pubera (Fr.) Sacc. Bipolar (Biggs, 1938).
P. roseo-cremeum (Bres.). Bipolar (Vandendries, 1937*b*).
P. versata Burt. Heterothallic (Biggs, 1938).
P. violacea-livida (Sommf.) Bres. Heterothallic (Biggs, 1938).
Solenia poriaeformis DC (Fr.) Heterothallic (Kniep, 1922).
Stereum hirsutum (Willd) Fr. Homothallic (Kniep, 1920).
S. murrayi (Berk. & Curt.) Burt. Heterothallic (Biggs, 1938).
S. purpureum (Pers.) Fr. Heterothallic (Kniep, 1920).
S. rufum Fr. Tetrapolar (Biggs, 1938).
Tomentella terrestris (Kniep). Homothallic (Kniep, 1913).

CLAVARIACEAE

Sparassis crispa (Fr.) Wulf. Bipolar (Fries, 1948).
Typhula erythropus Fr. Tetrapolar (Kniep, 1920, 1928; Lehfeldt, 1923).
T. gyrans (Batsch.) Fr. Heterothallic (Macdonald, 1934).
T. trifolii Rostr. Heterothallic (Noble, 1937).
T. sp. Homothallic (Kniep, 1920).

HYDNACEAE

Hydnum auriscalpium L. ex. Fr. Tetrapolar (7, 3) (13, 5) multiple alleles (Fries, 1941).
H. coralloides Scop. ex Fr. Tetrapolar (9, 9) multiple alleles (Fries, 1941).
H. corrugatum Fr. Tetrapolar (10, 6) multiple alleles (Fries, 1941).
H. strigosum Sw. ex Fr. Tetrapolar (8, 7) (Fries, 1941).
Irpex pendulus (A. & S.) Fr. Tetrapolar (5, 3) (Fries, 1941).
Odontia arguta (Fr.) Quél. Homothallic (Brown, 1935).
O. bicolor (A. & S.) Bres. Heterothallic (Brown, 1935).
O. fragilissima (B. & C.) Brown. Homothallic (Brown, 1935).
O. fusco-atra (Fr.) Bres. Homothallic (Brown, 1935).
O. fusco-atra (Fr.) Bres. Heterothallic (Biggs, 1938).
O. hydnoides (Cooke & Massee) v. Höhn. Homothallic (Brown, 1935).
O. separans (Peck.) Brown. Heterothallic (Brown, 1935).
O. setigera (Fr.) Mill. Bipolar (Biggs, 1938).
O. stenodon (Pers.) Bres. Homothallic (Brown, 1935).
O. sudans Burt. Tetrapolar (Biggs, 1938).
O. uda (Fr.) Bres. Homothallic (Brown, 1935).
Phlebia strigozonata (Schw.) Burt. Bipolar (Biggs, 1938).
Radulum orbiculare Fr. Tetrapolar (Biggs, 1938).
R. radula (Fr.) Nannf. (= R. orbiculare Grev. ex Fr.). Bipolar, multiple alleles (Fries, 1941).

POLYPORACEAE

Coriolus. *See* Polystictus.
Fomes igniarius (L.) Gill. Tetrapolar, multiple alleles (Verrall, 1937).
F. pinicola (Sw.) Cooke. Bipolar, multiple alleles (Mounce, 1926, 1929; Mounce & Macrae, 1938).
F. roseus (A. et S.) Cooke. Bipolar, multiple alleles (Mounce & Macrae, 1937).
F. subroseus (Weir) Overh. Bipolar, multiple alleles (Mounce & Macrae, 1937).
Lenzites betulina (L.) Fr. Tetrapolar (17, 8) (12, 4) multiple alleles (Vandendries & Brodie, 1933*a*, *b*; Vandendries, 1934*e*).
L. saepiaria (Wulf.) Fr. Bipolar, multiple alleles (Mounce & Macrae, 1936; Fries, 1936).
L. trabea (Pers.) Fr. (syn. L. thermophila Falck.). Bipolar, multiple alleles (Mounce & Macrae, 1936).
Leptoporus. *See* Polyporus.
Leucoporus. *See* Polyporus.
Melanopus. *See* Polyporus.
Polyporus abietinus (Dicks.) Fr. Tetrapolar (5, 3) multiple alleles (Fries, 1938; Raestad, 1941; Fries & Jonasson, 1941).
P. adustus (Willd.) Fr. Bipolar, multiple alleles (Vandendries, 1934*d*, 1936*b*).
P. arcularius (Batsch.) Fr. Tetrapolar (16, 7) (9, 6) (Vandendries, 1934*d*, 1936*c*).
P. betulinus (Bull.) Fr. Bipolar (Macdonald, 1937).
P. borealis (Wahlenb.) Fr. Tetrapolar (5, 7) (Robak, 1932).
P. brumalis (Pers.) Fr. Tetrapolar (Vandendries, 1934*d*, 1936*d*).
P. imberbis (Bull.) Quél. Bipolar (Vandendries, 1933*a*).
P. ostreiformis Berk. Bipolar (Bose, 1934).
P. palustris Berk. Bipolar, multiple alleles (Nobles, 1943).
P. squamosus (Huds.) Fr. Heterothallic, multiple alleles (Vandendries, 1934*d*, 1936*e*).
Polystictus hirsutus (Wulf.) Fr. Bipolar (Bose, 1934).
P. versicolor (L.) Fr. Tetrapolar (17, 13) (Kniep, 1920; Vandendries & Brodie, 1933*b*).
P. zonatus Fr. Tetrapolar (21, 7) (Vandendries & Brodie, 1933*b*).

Poria microspora Overh. Bipolar, multiple alleles (Nobles, 1943).
Trametes americana Overh. Bipolar, multiple alleles (Mounce & Macrae, 1936).
T. cinnabarina (Jacq.) Fr. Tetrapolar (11, 15) (4, 8), multiple alleles (Vandendries, 1934*a*; Fries, 1936).
T. hispida Bagl. Tetrapolar (18, 13) (Vandendries, 1934*a*).
T. serialis Fr. Bipolar, multiple alleles (Robak, 1936; Fries, 1936; Nobles, 1943).
T. suaveolens (L.) Fr. Tetrapolar (14, 5) (10, 9), multiple alleles (Vandendries, 1933*a*).

BOLETACEAE

Boletus variegatus (Fr.) Sw. Heterothallic (Fries, 1948).

AGARICACEAE

Agaricus campestris Fr. Homothallic (Klioushnikova, 1939).
A. campestris Fr. var. bisporus Klig. Homothallic (Kligman, 1943).
Agrocybe. *See* Naucoria, Pholiota.
Anellaria separata (L.) Karst. Bipolar (Vandendries, 1923*a*, *b*).
A. separata (L.) Karst. Tetrapolar (Routien, 1940).
Armillaria mucida (Schrad.) Fr. Tetrapolar (Kniep, 1920, 1928).
Clitocybe aurantiaca (Fr.) Wulf. Tetrapolar (5, 3), multiple alleles (Fries, 1948).
C. cyathiformis (Bull.) Fr. Bipolar (Vandendries, 1936*e*).
C. gallinacea (Scop.) Fr. Tetrapolar (Vandendries, 1936*e*).
C. infundibuliformis (Schaeff.) Fr. Heterothallic (Kniep, 1920).
Clitopilus pleurotelloides (Kühner). Homothallic, without clamp-connexions (Vandendries, 1936*f*, 1937*c*; Kühner & Vandendries, 1937).
C. pseudo-pinsitus (Kühner). Homothallic, without clamp-connexions (Vandendries, 1936*f*, 1937*c*, 1938*a*).
Collybia asema Fr. Heterothallic (Kniep, 1920).
C. butyracea (Bull.) Fr. Heterothallic (Kniep, 1920).
C. cirrhata (Schum.) Fr. Tetrapolar (20, 11), multiple alleles (Kniep, 1922, 1928; Arnold, 1935).
C. cookei (Bres.) Arn. Tetrapolar (11, 19) (Arnold, 1935).
C. tenacella Pers. Heterothallic, without clamp-connexions (Vandendries, 1936*f*, 1937*c*).
C. tuberosa (Bull.) Fr. Tetrapolar (14, 5) (14, 3), multiple alleles (Kniep, 1920; Harnack, 1931; Arnold, 1935).
C. velutipes (Curt.) Fr. Tetrapolar (8, 5) (13, 13) multiple alleles (Kniep, 1920; Vandendries, 1923*a*; Zattler, 1924; Funke, 1924; Newton, 1926*a*; Heldmaier, 1929).
Conocybe pubescens Gill. *See* Galera pubescens.
Coprinus asterophorus Long & Miller. Homothallic (Barnett, 1945).
C. auricomus Pat. Tetrapolar (12, 3), Quintanilha A. and L. & Vasermanis, 1941).
C. boudieri Quél. Bipolar (Quintanilha A. and L. & Vasermanis, 1941).
C. comatus Fr. Bipolar, multiple alleles (Mounce, 1921; Brunswik, 1924).
C. clavatus (Batt.) Quél. Heterothallic (Lendner, 1920).
C. curtus Kalchbr. Bipolar (Brunswik, 1924).
C. curtus Kalchbr. Tetrapolar (Newton, 1926*a*).
C. deliquescens Fr. Bipolar (Brunswik, 1924).
C. disseminatus (Pers.) Quél. *See* Psathyrella disseminata (Pers.) Fr.
C. domesticus (Pers.) Fr. Bipolar (Chow, 1934).
C. ephemeroides (Bull.) Fr. Homothallic, without clamp-connexions (Brunswik, 1924).
C. ephemerus (Bull.) Fr. Bipolar (Brunswik, 1924; Sass, 1929; Quintanilha, A and L. & Vasermanis, 1941).
C. ephemerus (Bull.) Fr. f. bisporus Sass. Secondarily homothallic (Sass, 1929).
C. erethistes Heim. Tetrapolar (8, 12) (Quintanilha A. and L. & Vasermanis, 1941).
C. extinctorius (Bull.) Fr. Tetrapolar (5, 5) (Quintanilha, 1941, 1944).
C. fimetarius (L.) Fr. *See also* C. lagopus Fr. Tetrapolar, multiple alleles (Bensaude, 1918; Kniep, 1922; Brunswik, 1924, 1926; Oort, 1930; Quintanilha, 1933, 1935, 1941).
C. fimetarius Fr. var. macrorhizus Pers. *See* C. macrorhizus (Pers.) Rea.
C. friesii Quél. Bipolar, multiple alleles (Brunswik, 1924).

Coprinus funariarum Métrod. Tetrapolar (10, 9) (Quintanilha, A. and L. & Vasermanis, 1941).
C. hiascens Fr. Bipolar (Quintanilha, 1941, 1944).
C. lagopus Fr. sensu Buller (syn. C. fimetarius (L.) Fr.). Tetrapolar, multiple alleles (Mounce, 1922; Hanna, 1925, 1928; Newton, 1926*b*; Buller, 1934).
C. lagopus Fr., sensu Brunswik (? not syn. C. fimetarius (L.) Fr.). Tetrapolar, multiple alleles (Brunswik, 1924).
C. macrorhizus (Pers.) Rea. Tetrapolar, multiple alleles (Dickson, 1936).
C. macrorhizus (Pers.) Rea. Bipolar (Routien, 1940).
C. micaceus (Bull.) Fr. Tetrapolar (12, 7), multiple alleles (Brunswik, 1924; Vandendries, 1926, 1927*a–c*, 1929*a*, *b*; Vandendries & Robyn, 1929; Routien, 1940).
C. narcoticus Fr. Homothallic (Mounce, in Buller, 1924*a*; Brunswik, 1924).
C. niveus (Pers.) Fr. Tetrapolar (13, 9) (5, 3), multiple alleles (Mounce, 1922; Brunswik, 1924).
C. picaceus (Bull.) Fr. Tetrapolar (26, 22) (8, 3), multiple alleles (Brunswik, 1924; Chow, 1934).
C. plagioporus Romagn. Homothallic (Quintanilha, 1941, 1944).
C. plicatilis Fr. Tetrapolar (Routien, 1940).
C. radians (Desm.) Fr. Bipolar, without clamp-connexions (Brunswik, 1924).
C. radians (Desm.) Fr. Bipolar, with clamp-connexions (Vandendries, 1924, 1925*a–d*).
C. radians (Desm.) Fr. Tetrapolar (6, 6) (Quintanilha, A. and L. & Vasermanis, 1941).
C. rostrupianus Hans. Bipolar, multiple alleles (Newton, in Buller, 1924*b*; Newton, 1926*a*).
C. semilantanus Peck. Tetrapolar (Routien, 1940).
C. sphaerosporus Dicks. Bipolar (Dickson, 1934).
C. stercorarius (Bull.) Fr. Homothallic (Mounce, 1921, 1922; Brunswik, 1924; Newton, 1926*a*).
C. stercorarius (Bull.) Fr. Heterothallic (Kniep, 1920; Vandendries, 1923*b*).
C. sterquilinus Fr. Homothallic (Lendner, 1920; Mounce, 1921, 1922; Brunswik, 1924; Harder, 1926; Newton, 1926*a*; Quintanilha, 1941).
C. tergiversans Fr. Bipolar (Vandendries, 1930*a*).
C. urticaecola (Berk. & Br.) Buller. Bipolar (Hanna, 1939).
C. velaris Fr. Bipolar (Brunswik, 1924).
Cortinellus berkeleyana Ito & Imai. Heterothallic (Nisikado & Yamauti, 1935).
Deconica coprophila. *See* Psilocybe coprophila Fr.
D. crobula (Fr.) Romagn. *See* Naucoria crobulus (Fr.) Quél.
D. inquilina (Fr.) Pat. *See* Naucoria inquilina (Fr.) Quél.
Drosophila. *See* Hypholoma.
Flammula carbonaria Fr. Tetrapolar (12, 7) (Quintanilha, A. and L. & Vasermanis, 1941).
Galera pubescens (Gill.). Tetrapolar (Vandendries, 1936*e*).
G. pubescens (Gill.) f. bispora. Secondarily homothallic (Vandendries, 1936*f*, 1937*c*).
G. tenera (Schaeff.) Fr. Tetrapolar, multiple alleles (Oikawa, 1939).
G. tenera (Schaeff.) f. semiglobata Kühner. Heterothallic (Vandendries, 1936*f*).
G. tenera (Schaeff.) Fr. f. bispora Sass. Secondarily homothallic (Sass, 1929).
Hypholoma appendiculata Fr. Tetrapolar (9, 14) (11, 8) (Quintanilha, A. and L. & Vasermanis, 1941).
H. atomata Fr. Heterothallic, multiple alleles (Quintanilha, 1941, 1944).
H. badia (Romagn.). Tetrapolar (5, 3), multiple alleles (Quintanilha, 1941, 1944).
H. candolleanum Fr. Tetrapolar (12, 18) (Kniep, 1920; Vandendries & Brodie, 1933*c*). Cross-fertile with H. appendiculata (Quintanilha, A. and L. & Vasermanis, 1941).
H. capnoides Fr. Tetrapolar (Kniep, 1920; Funke, 1924; Vandendries, 1937*b*).
H. caudata Fr. Tetrapolar (7, 7) (Quintanilha, 1941, 1944).
H. exalbicans (Romagn.). Tetrapolar (7, 13) (Quintanilha, A. and L. & Vasermanis, 1941).
H. fasciculare (Huds.) Fr. Tetrapolar (Kniep, 1920; Vandendries, 1922, 1923*a*; Funke, 1924).
H. fibrillosa (Pers.) Fr. Tetrapolar (19, 23) (Quintanilha, A. and L. & Vasermanis, 1941).
H. gracilis (Pers.) Fr. Tetrapolar (7, 9) (Quintanilha, A. and L. & Vasermanis, 1941; Quintanilha, 1944).
H. hydrophilum (Bull.) Fr. Tetrapolar (11, 8) (Kniep, 1920; Vandendries, 1930*d*).
H. marcescibilis Britz. Tetrapolar (12, 10) (Quintanilha, A. and L. & Vasermanis, 1941).
H. melantinum Fr. Tetrapolar (14, 10) (Vandendries & Brodie, 1933*b*).
H. prona Fr. Tetrapolar (13, 7) (14, 7) (8, 13), multiple alleles (Quintanilha, 1941, 1944).
H. semivestita Bk. Bipolar (Quintanilha, 1941, 1944).
H. subatrata (Bats.) Fr. Bipolar (Quintanilha, A. and L. & Vasermanis, 1941).

all of which are usually uninucleate (Fig. 13). In contrast with the observations of Palm and Fagerlind, the basal antipodal cell never showed more than a single nucleus. Nor was it found to differ appreciably from the other antipodal cells in size. But sometimes one or the other of the remaining antipodal cells may be binucleate. In Fig. 14 the second cell from the base has two nuclei and in Fig. 15 the third cell shows the same condition. Eventually, all of the antipodals degenerate forming a dark slender streak which is entirely absorbed during later development.

Since the main object of the present investigation was to study the development of the embryo sac, other observations are only incidental. It was noted, however, that as in other Compositae the nucellar epidermis disorganizes during the development of the embryo sac and the innermost layer of the integument becomes differentiated as the endothelium. A peculiar feature is that most of the cells of the anther tapetum degenerate *in situ*, and only a few protrude inside into the anther loculus. A true periplasmodium was never seen in our material.

It is concluded that the embryo sac of *Chrysanthemum parthenium* is tetrasporic and arises by two divisions of the megaspore nuclei, but there are differences with regard to the exact number of nuclei in the mature embryo sac and the organization of the antipodal cells. These differences may be related to environmental conditions or there may be different races of this species which behave somewhat differently from one another. Only further studies can settle this point.

REFERENCES

FAGERLIND, F. (1941). Die Embryosackentwicklung bei *Tanacetum vulgare* und einigen *Chrysanthemum*-Arten. *Svensk. Bot. Tidskr.* **35**, 157–76.

PALM, B. (1915). Studien über Konstruktionstypen und Entwicklungswege des Embryosackes der Angiospermen. Diss. Stockholm.

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A NEW APPROACH TO THE PROBLEM OF THE PRIMITIVE FLOWER

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In a previous paper (Sporne, 1948), the writer put forward the suggestion that correlations between floral and vegetative characters could be used in assessing the relative advancement of the families of Dicotyledons, and that this was the first essential before a natural system of classification could be drawn up. The correlations themselves were mentioned only briefly, and the present purpose is to set out the full details of these correlations, discussing the limitations of the information on which they are based and the reliability of the conclusions which can be drawn.

It is necessary first to consider theoretically the interpretation of these correlations. This was based on certain definitions which must be stated again in full. A primitive character is 'one which, possessed by some present-day families, was also possessed by their ancestors', and a primitive family is 'a present-day family which has retained a relatively large number of primitive characters and which has diverged very little from the ancestral'. Conversely, an advanced family is 'one which has diverged a long way from the ancestral type and which has retained relatively few primitive characters'. From this it follows that the overall distribution of any given primitive character is not a random one, but is displaced towards the primitive families. Consequently, any two primitive characters can be shown to occur together more frequently than they would if distributed at random throughout the flora. In other words, they tend to be correlated. In a similar way, the distribution of advanced characters is displaced towards the advanced families, with the result that advanced characters, too, tend to be correlated. The existence of a correlation between two characters, therefore, would suggest that they are both primitive or that they are both advanced. It must, nevertheless, be borne in mind that such a conclusion is not invariably justified, for if two characters were to depend for their expression on the same genetical or physiological factor then they, too, would tend to be correlated, regardless of their evolutionary significance. But it will be shown that at least twelve characters, some vegetative and some floral, are all intercorrelated, and it is most unlikely that they could all be determined by the same genetical or physiological factors.

There remains, however, the problem of deciding whether these characters are primitive or advanced, and at present this problem can only be resolved by reference to the fossil record. There are many lists of fossil remains of Dicotyledons, some of fossil wood, some of leaf-impressions, some of fruits and seeds, and some of pollen grains, but whichever list is selected for comparison with modern plants, certain doubts and misgivings persist. The first of these concerns correct identification, and while recent remains are the easiest to identify, they are the least useful for comparison. The earlier the remains, the better the comparison, but the harder it is to assign them correctly to a genus or a family. Also, such fossil remains as have been described represent but a small sample of the world vegetation of those early times, and one which is seldom likely to be a random sample.

Perhaps the least doubt is attached to the list of fossilized fruits and seeds found in the London Clay and listed by Reid & Chandler (1933). These remains are probably less liable to faulty identification than, for example, leaf impressions. Moreover, since there is no apparent reason why the fruits or seeds of herbaceous and of woody plants should not stand an equal chance of fossilization, there is no unwarranted bias towards woodiness. It can be taken that these remains are a random sample of a very local flora of Eocene times, but they are certainly not a representative sample of the world's flora of those times. The total number of families listed by Reid & Chandler is forty-one, most of which, being represented by a large number of specimens, are no doubt reliably identified. The identification of two families, Theaceae and Urticaceae, was, however, admitted to be doubtful, but evidence from other lists of fossil remains suggests that the Theaceae, at any rate, had representatives alive in Eocene times.

The ideal comparison would be between the floral or vegetative characters of modern families and those of the actual plants whose remains have been found, but clearly this is impracticable. It is only possible to compare modern representatives of these forty-one families with the complete flora of the world. When this is done, certain floral and vegetative characters stand out as occurring more frequently among these families than among the families of the world flora. Moreover, these characters are among the twelve which show intercorrelations among themselves. Such characters are therefore taken to be primitive rather than advanced.

A similar opportunity for comparison is afforded by the list of fossil wood compiled by Edwards (1931). In using this, it is necessary to compare the fossil families with such modern families as have woody members, rather than with the whole flora of the world. Clearly, herbaceous families were precluded by their very nature from being represented in a collection of fossil wood. This list contains forty-seven families of Dicotyledons whose woody remains have been identified from pre-Pleistocene deposits, and when modern representatives of these families are compared with all the woody families of the present day they show certain characters in common, some of which are among those shown by the London Clay families.

Together, then, these two lists of fossil remains supply evidence that evolutionary changes have occurred in the Dicotyledons, even though only a relatively short period of time has elapsed since the beginning of the Pleistocene age. It is true that these changes show up merely as changes in the frequency of characters, but they do act as pointers to the direction in which evolution is progressing, and they make it possible to distinguish the primitive from the advanced among characters which are intercorrelated.

THE CHARACTERS ON WHICH CORRELATIONS ARE BASED

Table 1 contains the 259 families of Dicotyledons recognized by Engler & Diels (1936), and in columns 1–12 the occurrence of twelve floral and vegetative characters is recorded as far as can be ascertained. Universal presence within a family of a certain character is indicated by ' + ', and complete absence by ' – '. The symbol ' ± ' indicates a 'mixed' family, i.e. one in which some genera or species exhibit the character while some do not. Where information is lacking a 'dot' is recorded. Frequently it happens that a character is described as being rarely present in a particular family, and hitherto the writer has recorded a ' – ' for such a family, i.e. excluding rarities. Such a course, however, gives scope for the accusation of bias in the exact interpretation of the various grades of rarity.

Table 1

	Trees or shrubs	Leaves glandular	Leaves alternate	Leaves stipulate	Flowers unisexual	Flowers actinomorphic	Petals free	Stamens pleiomerous	Carpels pleiomerous	Seeds arillate	Seeds with two integuments	Seeds with integument bundles	Proportion of advanced characters	Advancement index (%)
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Acanthaceae	±	±	—	—	—	±	—	—	—	—	—	—	10·5/12	88
Aceraceae	+	—	—	—	±	+	+	—	—	—	+	—	7·5/12	62
Achariaceae	+	—	+	—	+	+	—	—	—	—	+	—	7/12	58
Achatocarpaceae	+	—	+	.	+	+	.	+	—	—	+	—	4/10	40
Actinidiaceae	+	—	+	—	±	+	±	+	—	—	—	—	7/12	58
Adoxaceae	—	—	—	—	—	+	—	—	—	—	—	—	11/12	92
Aextoxicaceae	+	—	—	+	+	+	+	—	—	—	+	—	6/12	50
Aizoaceae	±	—	±	±	—	+	+	+	+	+	+	—	4·5/12	37
Akaniaceae	+	—	+	.	+	+	+	—	—	—	+	+	4/11	36
Alangiaceae	+	±	+	—	—	+	±	—	—	—	—	+	7/12	58
Amaranthaceae	—	—	±	—	±	+	.	—	—	+	+	—	7/11	64
Anacardiaceae	+	±	±	±	±	±	+	—	—	—	+	+	5·5/12	46
Ancistrocladaceae	+	—	+	+	—	+	—	—	—	—	+	—	7/12	58
Anonaceae	+	+	+	—	±	+	+	+	+	+	+	+	1·5/12	12
Apocynaceae	±	—	±	±	—	+	—	—	—	—	—	—	9·5/12	79
Aquifoliaceae	+	—	+	±	±	+	±	—	—	—	—	—	7·5/12	62
Araliaceae	+	—	+	+	±	+	±	—	—	—	—	—	7/12	58
Aristolochiaceae	±	—	+	—	—	±	.	+	—	+	+	+	5/11	45
Asclepiadaceae	±	—	±	—	—	+	—	—	—	—	—	—	10/12	83
Balanophoraceae	—	—	+	—	±	.	.	—	—	—	—	—	8·5/10	85
Balanopsidaceae	+	—	+	—	+	.	.	.	.	—	+	—	4/8	50
Balsaminaceae	—	±	±	—	—	—	±	—	—	—	+	—	9·5/12	79
Basellaceae	+	—	+	—	±	+	.	—	—	—	+	—	6·5/11	59
Batidaceae	+	—	—	—	+	—	.	—	—	—	+	—	8/11	73
Begoniaceae	±	—	+	+	+	±	+	+	—	—	+	—	5/12	42
Berberidaceae	±	—	+	±	—	+	+	—	—	+	+	—	6/12	50
Betulaceae	+	—	+	+	+	.	.	+	—	—	—	+	4/10	40
Bignoniaceae	±	—	±	—	—	—	—	—	—	—	—	—	11/12	92
Bixaceae	+	+	+	+	—	+	+	+	—	+	+	—	3/12	25
Bombacaceae	+	—	+	+	—	+	+	+	—	+	+	+	3/12	25
Boraginaceae	±	—	+	—	—	±	—	—	—	—	—	+	9/12	75
Bretschneideraceae	+	—	+	.	—	—	+	—	—	—	+	—	7/11	64
Brunelliaceae	+	—	—	+	+	+	.	—	—	—	+	—	6/11	55
Bruniaceae	+	—	+	—	—	±	±	—	—	—	—	—	9/12	75
Brunoniaceae	±	—	+	—	—	±	—	—	—	—	—	—	10/12	83
Burseraceae	+	±	±	±	±	+	±	—	—	—	+	—	6·5/12	54
Buxaceae	±	—	±	—	+	+	.	+	—	—	+	+	5/11	45
Byblidaceae	±	—	+	.	—	+	±	—	—	—	—	—	8/11	73
Cactaceae	±	—	.	.	—	±	+	+	—	—	+	—	6/10	60
Callitrichaceae	—	—	—	—	+	.	.	.	.	—	—	—	7/8	87
Calycanthaceae	+	+	—	—	—	+	.	+	+	—	+	—	5/11	45
Calyceraceae	—	—	+	—	±	±	—	—	—	—	—	—	10/12	83
Campanulaceae	±	—	±	—	—	±	±	—	—	—	—	—	10/12	83
Canellaceae	+	+	+	—	—	+	±	+	—	—	+	—	5·5/12	46
Capparidaceae	±	±	—	±	—	±	+	+	—	—	+	—	7/12	58
Caprifoliaceae	±	—	—	±	—	±	—	—	—	—	—	+	9·5/12	79
Caricaceae	+	—	+	—	±	+	—	—	—	—	+	+	6·5/12	54
Caryocaraceae	+	—	±	+	—	+	±	+	+	—	+	—	5/12	42
Caryophyllaceae	—	—	±	±	—	+	±	—	—	+	+	—	7·5/12	62
Casuarinaceae	+	—	—	—	+	.	.	.	.	—	+	+	4/8	50
Celastraceae	+	—	±	±	±	+	+	—	—	+	+	—	5·5/12	46
Cephalotaceae	—	—	+	—	—	+	.	—	—	—	+	—	8/11	73

Table 1 (*continued*)

	Trees or shrubs 1	Leaves glandular 2	Leaves alternate 3	Leaves stipulate 4	Flowers unisexual 5	Flowers actinomorphic 6	Petals free 7	Stamens pleiomerous 8	Carpels pleiomerous 9	Seeds arillate 10	Seeds with two integuments 11	Seeds with integument bundles 12	Proportion of advanced characters 13	Advancement index (%) 14
Ceratophyllaceae	−	−	−	−	+	+	.	+	−	−	−	−	8/11	73
Cercidiphyllaceae	+	−	−	+	+	+	.	+	−	−	+	−	5/11	45
Chenopodiaceae	±	−	±	−	±	±	.	−	−	−	±	−	8·5/11	77
Chlaenaceae	+	−	+	+	−	+	+	+	−	−	+	−	5/12	42
Chloranthaceae	±	+	−	+	±	.	.	−	−	−	−	−	7/10	70
Cistaceae	±	−	−	±	−	+	+	+	+	−	+	−	6/12	50
Clethraceae	+	−	+	−	−	+	+	−	−	−	−	−	8/12	67
Cneoraceae	+	−	+	−	−	+	+	−	−	−	+	−	7/12	58
Cochlospermaceae	+	±	+	+	−	±	+	+	−	−	+	−	5/12	42
Columelliaceae	+	−	−	−	−	±	−	−	−	−	−	−	10·5/12	88
Combretaceae	+	±	±	−	±	+	+	−	−	−	+	+	5·5/12	46
Compositae	±	±	±	±	±	±	−	−	−	−	−	+	8/12	67
Connaraceae	+	−	+	−	±	±	±	−	−	+	+	+	5·5/12	46
Convolvulaceae	±	−	+	±	−	+	−	−	−	−	−	−	9/12	75
Coriariaceae	+	−	−	−	±	+	+	−	−	−	+	−	7·5/12	62
Cornaceae	±	±	±	−	±	+	+	−	−	−	−	−	8/12	67
Corynocarpaceae	+	−	+	−	−	+	+	−	−	−	+	+	6/12	50
Crassulaceae	±	±	±	−	−	+	±	−	−	−	+	−	8/12	67
Crossosomataceae	+	−	+	.	−	+	+	+	+	−	+	−	4/11	36
Cruciferae	−	−	±	−	−	±	+	−	−	−	+	−	9/12	75
Crypteroniaceae	+	−	−	−	±	+	.	−	−	−	.	−	7·5/10	75
Cucurbitaceae	−	−	+	+	±	+	±	−	−	−	+	+	6/12	50
Cunoniaceae	+	−	−	+	±	+	+	+	−	−	+	−	5·5/12	46
Cynomoriaceae	−	−	+	−	±	.	.	−	−	−	−	−	8·5/10	85
Cyrillaceae	+	−	+	−	−	+	−	−	−	−	+	−	8/12	67
Daphniphyllaceae	+	−	+	−	+	+	.	−	−	−	+	−	6/11	55
Datiscaceae	±	−	+	−	±	+	+	+	−	−	+	−	6/12	50
Desfontainiaceae	+	−	−	.	−	+	−	−	−	−	−	−	9/11	82
Diapensiaceae	+	−	+	−	−	+	±	−	−	−	−	−	8·5/12	71
Dichapetalaceae	+	−	+	+	±	±	±	−	−	−	−	−	7·5/12	62
Diclidantheraceae	+	−	+	−	−	+	−	−	−	−	.	−	8/11	73
Didieraceae	+	−	+	−	+	+	+	+	−	−	+	−	5/12	42
Dilleniaceae	±	±	+	±	±	±	+	+	+	+	+	−	3·5/12	29
Dipsacaceae	−	−	−	−	−	−	−	−	−	−	−	−	12/12	100
Dipterocarpaceae	+	−	+	+	−	+	±	+	−	−	+	+	4·5/12	37
Droseraceae	−	−	+	+	−	+	+	−	−	−	+	−	7/12	58
Dysphaniaceae	−	−	+	−	±	+	.	−	−	−	.	−	7·5/10	75
Ebenaceae	+	−	+	−	±	+	−	−	+	−	+	+	5·5/12	46
Elaeagnaceae	+	−	±	−	±	+	.	−	−	−	+	−	7/11	64
Elaeocarpaceae	+	−	±	+	−	±	+	+	+	−	+	−	5/12	42
Elatinaceae	±	−	−	+	−	+	+	−	−	−	+	−	7·5/12	62
Empetraceae	+	−	+	−	+	+	+	−	+	−	−	−	6/12	50
Epacridaceae	+	−	±	−	±	+	−	−	−	−	−	−	9/12	75
Ericaceae	+	−	±	−	−	±	±	−	−	−	−	−	9·5/12	79
Erythroxylaceae	+	−	+	+	±	+	+	−	−	−	+	−	5·5/12	46
Eucommiaceae	+	−	+	−	+	+	.	.	.	−	.	−	4/8	50
Eucryphiaceae	+	−	−	+	−	+	+	+	+	−	+	−	5/12	42
Euphorbiaceae	±	±	±	±	+	+	±	+	±	+	+	+	3/12	25
Eupomatiaceae	+	−	+	−	−	+	.	+	+	−	+	−	5/11	45
Fagaceae	+	±	+	+	±	.	.	+	−	−	+	+	3/10	30
Flacourtiaceae	+	±	+	+	±	+	+	+	+	+	+	+	1/12	8
Fouquieraceae	+	−	+	−	−	+	−	−	−	−	+	−	8/12	67

Table 1 (*continued*)

	Trees or shrubs 1	Leaves glandular 2	Leaves alternate 3	Leaves stipulate 4	Flowers unisexual 5	Flowers actinomorphic 6	Petals free 7	Stamens pleiomerous 8	Carpels pleiomerous 9	Seeds arillate 10	Seeds with two integuments 11	Seeds with integument bundles 12	Proportion of advanced characters 13	Advancement index (%) 14
Frankeniaceae	±	—	—	—	—	+	+	+	—	—	+	—	7·5/12	62
Garryaceae	+	—	—	—	+	.	.	—	—	—	—	—	8/10	80
Geissolomataceae	+	—	—	—	—	+	.	—	—	—	+	—	8/11	73
Gentianaceae	±	—	—	—	±	±	—	—	—	—	—	±	10/12	83
Geraniaceae	±	±	±	±	—	±	+	—	—	—	+	—	7·5/12	62
Gesneriaceae	±	—	—	—	—	±	—	—	—	—	—	—	11/12	92
Globulariaceae	+	—	+	—	—	—	—	—	—	—	—	—	10/12	83
Gomortegaceae	+	—	—	—	—	+	.	—	—	—	+	—	8/11	73
Goodeniaceae	±	—	±	—	—	—	—	—	—	—	—	+	10/12	83
Grubbiaceae	+	—	—	—	—	+	.	—	—	—	—	—	9/11	82
Guttiferae	±	±	—	±	±	+	+	+	—	+	+	+	4/12	33
Gyrostemonaceae	+	—	+	.	+	+	.	+	+	—	—	—	4/10	40
Halorrhagaceae	±	—	±	—	±	+	+	—	—	—	+	—	7·5/12	62
Hamamelidaceae	+	—	±	+	±	±	+	—	—	—	+	—	6·5/12	54
Hernandiaceae	+	—	+	—	±	+	+	—	—	—	+	—	6·5/12	54
Heteropyxidaceae	+	—	±	—	±	+	+	—	—	—	.	—	7/11	64
Himantandraceae	+	—	+	—	—	.	.	+	—	—	+	+	5/10	50
Hippocastanaceae	+	—	—	—	±	—	+	—	—	—	+	+	7·5/12	62
Hippocrateaceae	+	—	±	±	—	+	+	—	—	—	+	—	7/12	58
Hippuridaceae	—	—	—	—	±	.	.	—	—	—	—	—	9·5/10	95
Hoplostigmataceae	+	—	+	.	—	+	—	+	—	—	—	—	7/11	64
Hydnoraceae	—	—	.	.	—	+	.	+	—	—	—	—	7/9	78
Hydrocaryaceae	—	—	—	—	—	+	+	—	—	—	+	+	8/12	67
Hydrophyllaceae	—	—	±	—	—	+	—	—	—	+	—	—	9·5/12	79
Hydrostachyaceae	—	—	.	.	+	.	.	.	.	—	—	—	5/6	83
Icacinaceae	+	—	+	—	±	+	±	—	—	—	+	—	7/12	58
Juglandaceae	+	—	+	—	+	.	.	+	—	—	—	+	5/10	50
Julianaceae	+	—	+	—	+	.	.	—	—	—	—	—	7/10	70
Labiatae	±	—	—	—	—	—	—	—	—	—	—	—	11·5/12	96
Lactoridaceae	+	—	+	+	+	+	.	—	—	—	+	—	5/11	45
Lardizabalaceae	+	—	+	—	±	+	+	—	—	—	+	—	6·5/12	54
Lauraceae	+	±	±	—	±	+	+	—	—	—	—	+	6·5/12	54
Lecythidaceae	+	—	+	—	±	+	±	+	+	—	.	+	4/11	36
Leguminosae	±	±	+	+	±	±	±	±	—	+	+	+	4/12	33
Leitneriaceae	+	—	+	—	+	—	.	.	.	—	+	—	5/9	56
Lennoaceae	—	—	.	—	—	+	—	—	±	—	—	—	9·5/11	86
Lentibulariaceae	—	—	+	—	—	—	—	—	—	—	—	—	11/12	92
Limnanthaceae	—	—	+	—	—	+	+	—	—	—	—	—	9/12	75
Linaceae	±	—	±	±	—	+	±	+	—	—	+	—	7/12	58
Lissocarpaceae	+	—	+	—	—	+	—	—	—	—	±	—	8·5/12	71
Loasaceae	+	—	±	—	—	+	±	+	±	—	—	—	7·5/12	62
Loganiaceae	+	—	—	±	±	+	—	—	—	—	—	—	9/12	75
Loranthaceae	+	—	—	—	±	±	±	—	—	—	—	—	9·5/12	79
Lythraceae	±	±	±	±	—	±	+	—	—	—	+	+	6·5/12	54
Magnoliaceae	+	+	+	±	±	+	+	+	+	+	+	±	1·5/12	12
Malesherbiaceae	±	—	+	—	—	+	—	—	—	—	+	—	8·5/12	71
Malpighiaceae	+	—	—	±	—	±	+	—	—	—	+	—	8/12	67
Malvaceae	±	—	+	+	±	+	+	+	+	—	+	+	3/12	25
Marcgraviaceae	+	—	+	—	—	+	+	+	+	—	+	—	5/12	42
Martyniaceae	—	—	—	—	—	—	—	—	—	—	—	—	12/12	100
Medusagynaceae	+	—	—	—	—	+	+	+	+	—	.	—	6/11	55
Melastomataceae	±	—	—	—	—	+	±	—	+	+	+	—	7/12	58

Table 1 (*continued*)

	Trees or shrubs 1	Leaves glandular 2	Leaves alternate 3	Leaves stipulate 4	Flowers unisexual 5	Flowers actinomorphic 6	Petals free 7	Stamens pleiomerous 8	Carpels pleiomerous 9	Seeds arillate 10	Seeds with two integuments 11	Seeds with integument bundles 12	Proportion of advanced characters 13	Advancement index (%) 14
Meliaceae	+	+	+	−	−	+	±	−	−	+	+	−	5·5/12	46
Melianthaceae	+	−	+	±	±	−	+	−	−	+	+	−	6/12	50
Menispermaceae	+	−	+	−	+	±	±	±	±	−	+	−	6/12	50
Monimiaceae	+	+	−	−	±	±	+	+	+	−	+	−	5/12	42
Moraceae	+	−	±	+	+	+	.	−	−	−	+	−	5·5/11	50
Moringaceae	+	−	+	−	−	−	+	−	−	−	+	+	7/12	58
Myoporaceae	+	±	±	−	−	±	−	−	±	−	−	−	9/12	75
Myricaceae	+	±	+	±	+	.	.	.	.	−	−	+	3/8	37
Myristicaceae	+	+	+	−	+	+	.	+	−	+	+	+	2/11	18
Myrothamnaceae	+	−	−	+	+	+	.	.	.	−	+	−	4/9	44
Myrsinaceae	+	±	+	−	±	+	±	−	−	−	+	−	6·5/12	54
Myrtaceae	+	±	±	±	±	+	+	+	+	−	+	+	3/12	25
Myzodendraceae	+	−	+	−	+	.	.	−	−	−	−	−	7/10	70
Nepenthaceae	+	−	+	−	+	+	.	+	−	−	+	−	5/11	45
Nolanaceae	±	−	+	−	−	+	−	−	−	−	−	−	9·5/12	79
Nyctaginaceae	±	−	±	−	±	+	.	−	−	−	+	−	7·5/11	68
Nymphaeaceae	−	±	+	±	−	+	+	+	+	+	+	±	3·5/12	29
Nyssaceae	+	±	+	−	±	+	+	−	±	−	+	−	5·5/12	46
Ochnaceae	±	−	+	+	−	±	+	+	±	−	±	+	5/12	42
Octoknemataceae	+	−	+	−	+	+	.	−	−	−	±	−	6·5/11	59
Olacaceae	+	±	+	−	−	+	±	−	−	−	±	−	6·5/12	54
Oleaceae	+	−	−	−	±	+	±	−	−	−	−	+	8/12	67
Oliniaceae	+	−	−	−	−	+	+	−	−	−	+	−	8/12	67
Onagraceae	±	−	±	±	−	±	+	−	−	−	+	−	8/12	67
Opiliaceae	+	−	+	−	±	+	±	−	−	−	−	−	8/12	67
Orobanchaceae	−	−	+	−	−	−	−	−	−	−	−	−	11/12	92
Oxalidaceae	±	−	+	±	−	+	±	−	−	+	+	−	6·5/12	54
Pandaceae	+	−	+	+	+	+	+	−	−	−	.	−	5/11	45
Papaveraceae	±	−	±	±	−	±	+	+	+	−	+	−	6/12	50
Passifloraceae	±	−	+	±	±	+	+	−	−	+	+	−	5·5/12	46
Pedaliaceae	−	−	±	−	−	−	−	−	−	−	−	−	11·5/12	96
Penaeaceae	+	−	−	±	−	+	.	−	−	−	+	−	7·5/12	62
Pentaphyllacaceae	+	−	+	−	−	+	+	−	−	−	+	−	7/12	58
Phrymaceae	−	−	−	−	−	−	−	−	−	−	−	−	12/12	100
Phytolaccaceae	±	±	±	±	±	+	.	±	+	+	+	−	4/11	36
Piperaceae	±	+	±	±	±	.	.	−	±	−	+	−	5·5/10	55
Pirolaceae	−	−	+	−	−	+	±	−	−	−	−	−	9·5/12	79
Pittosporaceae	+	−	±	−	±	+	+	−	−	−	−	−	8/12	67
Plantaginaceae	−	−	±	−	±	+	−	−	−	−	−	−	10/12	83
Platanaceae	+	−	+	+	+	+	+	−	±	−	+	−	4·5/12	37
Plumbaginaceae	±	−	+	−	−	+	±	−	−	−	+	−	8/12	67
Podostemonaceae	−	−	.	.	−	±	.	+	−	−	+	−	6·5/9	72
Polemoniaceae	−	−	±	−	−	±	−	−	−	−	−	−	11/12	92
Polygalaceae	±	±	+	−	−	−	±	−	−	+	+	+	6·5/12	54
Polygonaceae	±	±	±	+	±	+	.	−	−	−	±	−	6·5/11	59
Portulacaceae	±	±	±	+	−	+	±	±	±	−	+	−	6/12	50
Primulaceae	−	±	±	−	−	±	−	−	−	−	+	−	9·5/12	79
Proteaceae	+	−	±	−	±	±	.	−	−	−	+	−	7·5/11	68
Punicaceae	+	−	+	−	−	+	+	+	+	−	+	−	5/12	42
Quiinaceae	+	−	−	+	±	+	+	+	±	−	+	−	5/12	42
Rafflesiaceae	−	−	.	−	±	+	.	+	−	−	±	−	7/10	70
Ranunculaceae	−	−	±	±	±	±	+	+	+	+	±	−	5·5/12	46

Table 1 (*continued*)

	Trees or shrubs 1	Leaves glandular 2	Leaves alternate 3	Leaves stipulate 4	Flowers unisexual 5	Flowers actinomorphic 6	Petals free 7	Stamens pleiomerous 8	Carpels pleiomerous 9	Seeds arillate 10	Seeds with two integuments 11	Seeds with integument bundles 12	Proportion of advanced characters 13	Advancement index (%) 14
Resedaceae	—	—	+	+	±	+	+	+	—	+	+	—	4·5/12	37
Rhamnaceae	±	±	±	+	±	+	+	—	—	+	+	+	4/12	33
Rhizophoraceae	+	—	±	+	—	+	+	+	—	+	+	+	3·5/12	29
Rhoipteleaceae	+	—	—	+	±	+	.	.	.	—	+	—	4·5/9	50
Roridulaceae	±	—	+	—	—	+	—	—	—	—	+	—	8·5/12	71
Rosaceae	±	—	±	±	—	+	+	±	±	—	+	+	5·5/12	46
Rubiaceae	±	±	—	+	+	±	—	—	—	—	—	+	8/12	67
Rutaceae	±	±	±	±	±	±	±	—	±	—	+	—	7/12	58
Sabiaceae	+	±	+	—	±	+	+	—	—	—	+	—	7/12	58
Salicaceae	+	—	+	+	+	.	.	+	.	—	+	—	3/9	33
Salvadoraceae	+	—	—	+	±	+	+	—	—	—	+	—	7/12	58
Santalaceae	±	±	±	—	±	+	.	—	—	—	—	—	8/11	73
Sapindaceae	+	±	+	±	±	±	+	±	—	+	+	+	3·5/12	29
Sapotaceae	+	—	+	±	—	+	—	—	±	—	—	+	7/12	58
Sarraceniaceae	—	—	+	—	—	+	+	+	—	—	—	—	8/12	67
Saururaceae	—	—	+	+	—	.	.	.	.	—	+	—	5/8	62
Saxifragaceae	±	±	+	±	±	±	±	—	—	+	±	—	6·5/12	54
Scrophulariaceae	±	—	±	—	—	—	—	—	—	—	—	—	11/12	92
Scytopetalaceae	+	—	+	—	—	+	—	+	—	—	—	+	7/12	58
Simarubaceae	+	±	±	—	±	+	±	—	—	—	+	+	6/12	50
Solanaceae	±	—	+	—	—	±	—	—	—	—	—	—	10/12	83
Sonneratiaceae	+	—	—	—	±	+	+	+	+	—	+	—	5·5/12	46
Stachyuraceae	+	—	+	+	±	+	+	—	—	+	+	—	4·5/12	37
Stackhousiaceae	—	—	+	—	—	+	+	—	—	—	—	—	9/12	75
Staphyleaceae	+	—	—	+	±	+	+	—	—	—	+	+	5·5/12	46
Sterculiaceae	+	—	±	+	±	+	±	+	—	—	+	+	4·5/12	37
Strasburgeriaceae	+	—	+	—	—	+	±	—	—	—	.	—	7·5/11	68
Stylidiaceae	±	—	+	—	±	±	±	—	—	—	—	—	9/12	75
Styracaceae	+	—	+	—	—	+	±	—	—	—	±	+	7/12	58
Symplocaceae	+	—	+	—	—	+	±	—	—	—	±	—	8/12	67
Tamaricaceae	+	—	+	—	—	+	+	±	—	—	+	—	6·5/12	54
Theaceae	+	±	±	—	±	+	±	+	+	—	+	+	4/12	33
Thelygonaceae	—	—	±	+	+	.	.	+	—	—	—	—	6·5/10	65
Theophrastaceae	+	—	+	—	±	+	—	—	—	—	+	—	7·5/12	62
Thymelaeaceae	±	±	±	—	±	+	+	+	—	+	+	+	4/12	33
Tiliaceae	±	—	±	±	±	+	+	+	+	+	+	—	4/12	33
Tovariaceae	—	—	+	—	—	+	+	—	—	—	+	—	8/12	67
Tremandraceae	+	—	±	—	—	+	+	—	—	+	+	—	6·5/12	54
Trigoniaceae	+	—	±	±	—	—	+	—	—	—	+	—	8/12	67
Trochodendraceae	+	—	—	±	±	+	.	+	+	—	+	+	4/11	36
Tropaeolaceae	—	±	±	±	—	—	+	—	—	—	+	—	8·5/12	71
Turneraceae	±	—	+	—	—	+	+	—	—	+	+	—	6·5/12	54
Ulmaceae	+	—	+	+	±	+	.	—	—	—	+	—	5·5/11	50
Umbelliferae	—	—	+	—	—	±	+	—	—	—	—	—	9·5/12	79
Urticaceae	±	±	±	+	±	+	.	—	—	—	+	—	6/11	55
Valerianaceae	—	—	—	—	±	—	—	—	—	—	—	—	11·5/12	96
Verbenaceae	±	±	—	—	—	±	—	—	—	—	—	—	10·5/12	88
Violaceae	±	±	+	+	—	±	±	—	—	+	+	—	6/12	50
Vitaceae	+	±	+	±	±	+	±	—	—	—	+	+	5/12	42
Vochysiaceae	+	—	—	±	—	—	+	—	—	—	+	—	8·5/12	71
Zygophyllaceae	±	—	+	+	—	±	+	—	—	—	+	—	7/12	58

Furthermore, the size of a family affects the numerical importance of a few peculiar species within it. Thus, one species might represent 50% of a small family and only 0·03% of a large family like the Labiatae. It has therefore been decided to include rarities and to record a '+' in all such cases. As a result, all correlations have been recalculated, and while many new correlations have been found to occur, a few are no longer significant. Similarly, the advancement index has been recalculated for each family, with the result that the figures quoted here are slightly different from those given heretofore.

Column 1 contains six such examples of the inclusion of a rarity, in the families Campanulaceae, Gentianaceae, Labiatae, Onagraceae, Papaveraceae and Stylidiaceae. All these families are herbs except for very occasional woody members and have, until now, been treated as herbaceous. They are now, however, treated as mixed families, and the effect of this in the case of the Labiatae, for example, is to reduce its advancement index from 100 to 96.

The information quoted in column 2 is from Blenk (1884), and concerns those types of glands which are visible as translucent spots in the leaves. It may be that such a criterion allows the inclusion of glands of several different types of anatomical construction, but no attempt has been made to distinguish such types. It is interesting to find that, even so, a large number of correlations are shown, both with other characters and with the fossil record.

The recording of leaf-arrangement in column 3 is almost complete, though such families as Cactaceae, Hydnoraceae, and Podostemonaceae are left blank, because the leaves are so modified that their arrangement is open to question. For the same reason, there are some gaps in the information about stipules in column 4, though a few are also due to lack of information in the literature.

Under the heading of 'flowers unisexual' in column 5, no attempt has been made to distinguish those families in which unisexual flowers have been derived from bisexual and those in which unisexuality might be fundamental. To attempt such a distinction would be to introduce subjective reasoning into a process which is otherwise as far as possible objective. For although few would question that unisexual flowers with rudiments of the opposite sex have evolved from bisexual, it is a matter for speculation when such rudiments are absent. Yet, notwithstanding, unisexual flowers treated as they are here, still show correlations which lead to the conclusion that the primitive flower is unisexual. Clearly, were it possible to exclude from such correlations all families with derived unisexuality, their significance would be greater.

Similar points occur in the recording of the perianth symmetry. Flowers with neither petals nor sepals might have evolved from actinomorphic or from zygomorphic ancestors, or from apetalous and asepalous ancestors. Hence, for calculations concerning perianth symmetry, such families must be excluded until more is known about their ancestry. In column 7, for similar reasons, blanks are recorded for all families lacking a corolla, for an apetalous family could conceivably have been evolved equally easily from a gamopetalous or a polypetalous ancestor, though all the evidence suggests that apetaly is itself a primitive character.

'Pleiomery of the androecium', as recorded in column 8, is taken to include all cases where the number of stamens is described as $> 2n$ or as ∞ (where n = number of petals or sepals). In certain cases, therefore, where petals and sepals are both absent, and the number of stamens is limited, the entry is blank. Similar considerations arise in the next column, though here 'pleiomery of gynoecium' is taken to include all cases where the number of carpels is described as $> n$ or as ∞.

Information about the presence of an aril, listed in column 10, is derived from Willis (1931), Netolitzky (1926), Engler & Prantl (1887), and Engler & Diels (1936). The idea that arils should be regarded as primitive was first suggested to the writer by Corner,* and the large number of correlations shown by this feature supports the idea. The occurrence of vascular bundles in the integuments of the ovules has been listed by Le Monnier (1872) and by Netolitzky, and from the latter source came also some of the information concerning the number of integuments, though most was taken from Engler & Prantl or Engler & Diels.

CORRELATIONS

Each of the twelve characters listed in Table 1 has been considered in conjunction with the remaining eleven, and Pearson's χ^2 test has been applied, as explained by Fisher (1936), to determine where correlations occur. The results are set out in Table 2, where the following symbols are used throughout. In each correlation the first character referred to is character 'X' and the second character 'Y'. The letter 'e.', when used, indicates that 'mixed' families are excluded from the count, i.e. the only families to be included are those in which all species show the character. The letters 'n.e.' indicate that mixed families are included (even those in which the character rarely occurs). Although tests have been made using first exclusive figures, and then inclusive figures, only the most significant of the four, in each case, is listed here:

n = number of families on which the correlation is based, (i.e. 'total population');

x = number of families exhibiting character X;

y = number of families exhibiting character Y;

m = number of families exhibiting both characters X and Y;

$m_1 = \frac{xy}{n}$ = expected number of families exhibiting both characters, assuming them to be distributed at random among the n families.

The value of χ^2 is then calculated as follows:

$$\chi^2 = \frac{(m-m_1)^2}{x-m_1} + \frac{(m-m_1)^2}{y-m_1} + \frac{(m-m_1)^2}{m_1} + \frac{(m-m_1)^2}{n+m_1-x-y}.$$

The value of 'p'† which corresponds to this value of χ^2 indicates the mathematical probability that the correlation is not a genuine one, and that the figure obtained for 'm' is merely fortuitous. Thus a value of $p=0{\cdot}02$ would indicate a 2% probability that the correlation is not significant. This value of 'p' is set as the threshold value, and any correlation where p is greater than 0·02 is regarded as not significant. The observed occurrence 'm' of the two characters may be greater or less than the calculated occurrence 'm_1', but in both cases the value for χ^2 is positive, since it is the square of the difference $(m-m_1)$ which is used in the calculation. In the former case, the correlation is described as positive, since the two characters occur together more often than would be expected, and in the latter case as negative, since they occur together less often than would be expected.

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* It is understood that a paper concerning the significance of arils is being published by E. J. H. Corner in *Ann. Bot., Lond.*

† The corresponding values of 'p' and 'χ^2' for any given number of degrees of freedom can be found from mathematical tables. In all cases quoted here, the number of degrees of freedom is one.

Table 2

Serial no.	Character 'X'	Character 'Y'	n	x	y	m	m_1	χ^2	p	Type of correlation
1	Integument bundles (n.e.)	Trees or shrubs (n.e.)	259	58	208	54	46·6	7·7	<0·01	+
2	,, ,, (n.e.)	Glandular leaves (n.e.)	259	58	57	25	12·8	19·2	<0·001	+
3	,, ,, (n.e.)	Flowers unisexual (n.e.)	259	58	132	38	23·5	19·3	<0·001	+
4	,, ,, (n.e.)	Stamens pleiomerous (n.e.)	248	56	76	28	17·2	12·6	<0·001	+
5	,, ,, (n.e.)	Seeds arillate (n.e.)	259	58	35	17	7·8	16·2	<0·001	+
6	Two integuments (e.)	Trees or shrubs (e.)	250	156	136	98	84·9	11·8	<0·001	+
7	,, ,, (n.e.)	Glandular leaves (n.e.)	250	167	57	46	38·1	6·2	<0·02	+
8	,, ,, (e.)	Stipules present (n.e.)	238	150	97	79	61·1	23·9	≪0·001	+
9	,, ,, (e.)	Flowers actinomorphic (e.)	230	149	156	112	101·0	10·6	<0·01	+
10	,, ,, (e.)	Petals free (e.)	191	119	91	80	56·7	48·9	≪0·001	+
11	,, ,, (e.)	Stamens pleiomerous (n.e.)	240	149	74	60	45·9	20·3	≪0·001	+
12	,, ,, (e.)	Stamens meiomerous (n.e.)	240	149	25	5	15·5	20·9	≪0·001	−
13	,, ,, (e.)	Carpels pleiomerous (n.e.)	240	149	43	35	26·7	8·1	<0·01	+
14	,, ,, (e.)	Carpels meiomerous (e.)	240	149	119	60	73·9	13·7	<0·001	−
15	,, ,, (n.e.)	Seeds arillate (n.e.)	250	167	36	35	24·0	17·7	<0·001	+
16	Seeds arillate (n.e.)	Glandular leaves (n.e.)	259	35	57	17	7·7	16·7	<0·001	+
17	,, ,, (n.e.)	Leaves alternate (n.e.)	253	35	195	33	27·0	6·8	<0·01	+
18	,, ,, (n.e.)	Stipules present (n.e.)	247	35	98	24	13·9	14·2	<0·001	+
19	,, ,, (n.e.)	Petals free (n.e.)	197	33	145	33	24·3	14·2	<0·001	+
20	,, ,, (n.e.)	Stamens pleiomerous (n.e.)	248	35	76	21	10·7	16·6	<0·001	+
21	Carpels meiomerous (e.)	Trees or shrubs (n.e.)	248	123	199	91	98·7	6·0	<0·02	−
22	,, pleiomerous (n.e.)	Stipules present (n.e.)	237	43	93	25	16·9	7·8	<0·01	+
23	,, meiomerous (e.)	Flowers actinomorphic (e.)	235	113	162	62	77·9	17·5	<0·001	−
24	,, pleiomerous (n.e.)	Petals free (n.e.)	197	39	145	35	28·7	6·5	<0·02	+
25	,, meiomerous (e.)	Petals free (n.e.)	197	91	145	53	67·0	20·6	≪0·001	−
26	Stamens pleiomerous (n.e.)	Carpels meiomerous (e.)	248	76	123	22	37·7	18·7	<0·001	−
27	,, pleiomerous (n.e.)	Carpels pleiomerous (n.e.)	248	76	45	35	13·8	57·4	≪0·001	+
28	,, meiomerous (n.e.)	Carpels meiomerous (e.)	248	25	123	25	12·4	28·2	≪0·001	+
29	,, meiomerous (n.e.)	Trees or shrubs (n.e.)	248	25	202	14	20·4	9·6*	<0·001	−

30	Stamens meiomerous (n.e.)	Leaves alternate (n.e.)	243	25	189	12	19·4	14·1	<0·001	−
31	" meiomerous (n.e.)	Stipules present (n.e.)	237	25	93	3	9·3	8·7	<0·01	−
32	" pleiomerous (n.e.)	Stipules present (n.e.)	237	69	93	40	27·0	14·5	<0·001	+
33	" meiomerous (n.e.)	Flowers actinomorphic (e.)	235	22	162	5	15·2	21·1*	≪0·001	−
34	" pleiomerous (n.e.)	Petals free (n.e.)	197	56	145	53	41·2	17·7	≪0·001	+
35	" meiomerous (n.e.)	Petals free (n.e.)	197	19	145	4	13·9	29·7	≪0·001	−
36	Petals free (n.e.)	Trees or shrubs (n.e.)	197	145	160	124	117·8	6·6	<0·02	+
37	" " (n.e.)	Leaves glandular (n.e.)	197	145	47	41	34·6	5·7	<0·02	+
38	" " (n.e.)	Stipules present (n.e.)	190	140	78	70	57·5	17·5	<0·001	+
39	" " (n.e.)	Flowers actinomorphic (e.)	197	145	129	104	94·9	9·3	<0·01	+
40	Flowers actinomorphic (e.)	Trees or shrubs (e.)	239	165	133	108	91·8	21·3	≪0·001	+
41	" actinomorphic (e.)	Flowers unisexual (n.e.)	239	165	114	88	79·4	5·8	<0·02	+
42	" unisexual (e.)	Trees or shrubs (e.)	259	38	144	31	21·1	12·2	<0·001	+
43	Stipules present (n.e.)	Leaves glandular (n.e.)	247	98	57	32	22·6	8·4	<0·01	+
44	Leaves alternate (e.)	Trees or shrubs (e.)	253	127	144	84	72·3	8·8	<0·01	+
45	London Clay (Eocene)	Integument bundles (n.e.)	259	41	58	17	9·2	10·2	<0·01	+
46	" " "	Seeds arillate (n.e.)	259	41	35	12	5·5	10·5	<0·01	+
47	" " "	Carpels meiomerous (e.)	248	41	123	11	20·3	10·0	<0·01	−
48	" " "	Carpels pleiomerous (n.e.)	248	41	45	14	7·4	8·5	<0·01	+
49	" " "	Flowers unisexual (n.e.)	259	41	132	30	20·9	9·6	<0·01	+
50	" " "	Stipules present (n.e.)	247	41	98	24	16·3	7·2	<0·01	+
51	" " "	Leaves alternate (n.e.)	254	41	195	40	31·5	12·8	<0·001	+
52	" " "	Leaves glandular (n.e.)	259	41	57	23	9·02	33·1	≪0·001	+
53	" " "	Trees or shrubs (n.e.)	259	41	208	40	32·9	10·2	<0·01	+
54	Pre-Pleistocene Wood	Integument bundles (n.e.)	219	47	55	24	11·8	21·3	≪0·001	+
55	" "	Two integuments (e.)	211	41	143	35	27·8	7·2	<0·01	+
56	" "	Flowers unisexual (n.e.)	219	47	117	39	25·1	21·0	≪0·001	+
57	" "	Stipules present (n.e.)	210	47	91	29	20·1	8·9	<0·01	+
58	" "	Leaves glandular (n.e.)	219	47	53	22	11·4	16·6	<0·001	+

* Yate's correction for small numbers has been applied in correlations 29 and 33, as explained by Fisher (1936).

The first forty-four correlations listed in Table 2 are intercorrelations between characters, while the remaining fourteen are comparisons of fossil floras with the present-day flora. In the latter case, for the purposes of statistical calculation, occurrence in the fossil flora is treated exactly as a character 'X'. It will be noticed that the merism of the androecium and of the gynoecium is treated in two different ways, in the first instance, as already explained, under 'stamens pleiomerous' and 'carpels pleiomerous'; in the second place, allowance has been made for isomerous flowers by calculations under the headings 'stamens meiomerous' and 'carpels meiomerous'.

Table 3

	Trees or shrubs	Leaves glandular	Leaves alternate	Leaves stipulate	Flowers unisexual	Flowers actinomorphic	Petals free	Stamens pleiomerous	Stamens not meiomerous	Carpels pleiomerous	Carpels not meiomerous	Seeds arillate	Seeds with two integuments	Seeds with integument bundles	London Clay	Pre-Pleistocene wood
Seeds with integument bundles	**+**	**+**	··⁝··	··⁝··	**+**	⊙	··⁝··	**+**	··⁝··	··⁝··	··⁝··	**+**	··⁝··		**+**	**+**
Seeds with two integuments	**+**	**+**	··⁝··	**+**	··⁝··	**+**	**+**	**+**	**+**	**+**	**+**	**+**			··⁝··	**+**
Seeds arillate	··⁝··	**+**	**+**	**+**	··⁝··	⊙	**+**	**+**	··⁝··	··⁝··	··⁝··				**+**	··⁝··
Carpels not meiomerous	**+**	··⁝··	··⁝··	··⁝··	⊙	**+**	**+**	**+**	**+**	▨					**+**	··⁝··
Carpels pleiomerous	··⁝··	··⁝··	····	**+**	··⁝··	··⁝··	**+**	**+**	··⁝··						**+**	··⁝··
Stamens not meiomerous	**+**	··⁝··	**+**	**+**	··⁝··	**+**	**+**	▨							··⁝··	··⁝··
Stamens pleiomerous	··⁝··	··⁝··	··⁝··	**+**	··⁝··	··⁝··	**+**								··⁝··	····
Petals free	**+**	**+**	··⁝··	**+**	··⁝··	**+**									··⁝··	··⁝··
Flowers actinomorphic	**+**	··⁝··	··⁝··	··⁝··	**+**										····	··⁝··
Flowers unisexual	**+**	··⁝··	··⁝··	··⁝··											**+**	**+**
Leaves stipulate	··⁝··	**+**	⊙												**+**	**+**
Leaves alternate	**+**	··⁝··													**+**	··⁝··
Leaves glandular	····														**+**	**+**
Trees or shrubs															**+**	▨

The conclusions from Table 2 have been summarized in Table 3 by the insertion of the appropriate symbol in heavy type wherever a significant correlation occurs. Where a meaningless correlation occurs, the appropriate square is blocked in. Thus, it would be ridiculous to take account of a correlation between 'stamens not meiomerous' and 'stamens pleiomerous' (and similarly for carpels). These squares are therefore blocked in, and so is the one corresponding to the correlation between the possession of a woody habit and occurrence in the list of pre-Pleistocene fossil woods. In cases where the observed and the calculated figures are identical the symbol '⊙' is inserted in the appropriate square. The remaining squares correspond to correlations in which there is a difference between the observed and the calculated figures, though the difference is not large enough to bring the value of 'p' to less than 0·02. The symbol '··⁝··' indicates that the observed is greater than the calculated and the symbol '····' that it is less.

Some explanation should also be given of the apparent discrepancy between Tables 2 and 3 in the wording of the correlations concerning meiomery of androecium and gynoecium. In all cases involving these characters in Table 2, negative correlations are obtained with primitive characters. In transferring the information to Table 3, the wording has been changed so that the negative correlation is implied in the heading. Thus, a negative correlation with 'carpels meiomerous' is expressed as a positive correlation with 'carpels not meiomerous'.

The results are remarkable not only for the large number of significant positive correlations which are shown to exist, but also for the fact that there is not a single significant negative correlation. Indeed, among the 89 squares within the triangle in Table 3, only two '····' signs occur and only four '⊙' signs. The remainder are all either '··⁝··' or '+'. Now, although each '··⁝··' sign represents a correlation which by itself is not significant, when considered together the large number which occur lend very strong support to the forty-four significant correlations. There can be little doubt that the characters concerned are primitive.

THE PRIMITIVE FLOWERING PLANT

We may therefore picture the ancestral Dicotyledon as a tree or a shrub with alternate, glandular, stipulate leaves. Its flowers were unisexual, actinomorphic, and polypetalous; it had many stamens and many carpels; its seeds had two vascularized integuments outside which was an aril. Many of these characters are already regarded by morphologists as primitive, but it is, nevertheless, satisfactory to find evidence for such conclusions.

In the past there has been some discussion as to whether the tree habit is primitive or not. While the majority of botanists would agree that the arboreal habit is really primitive, there are some who claim that it is retrograde, and that the ancestral Dicotyledon was herbaceous. Hutchinson (1926), on the other hand, believes that the herbaceous and the woody represent two parallel lines of evolution, but correlations similar to those already described, even when based on Hutchinson's families, point nevertheless to the same conclusion, that the arboreal habit is in fact primitive and the herbaceous advanced.

Even more discussion has centred around the unisexual flower. On the one hand, there are those who favour the catkin as the ancestral type, and on the other those who favour the Ranalian flower. Engler & Prantl held the former view, and more recently Rendle (1925) also stated his belief in the haplochlamydeous unisexual flower as the primitive type. But the evidence of correlations, while supporting this claim for unisexuality, is less convincing as regards haplochlamydy. There is a high degree of correlation between these two characters, yet haplochlamydy shows insignificant correlations with the other eleven primitive characters. Hence, although it probably is primitive, its inclusion in the list of primitive characters is not yet justified.

The Gamopetalae have long been regarded as the most advanced group of Dicotyledons, and whereas originally fusion of petals was taken to indicate the close relationship of the families within the Gamopetalae, it is now generally realized that the group is heterogeneous, and that the fusion of petals merely indicates advancement. It is no surprise, therefore, to find correlations which indicate that among families which possess a corolla, those with free petals are primitive. Nor is it surprising to find that actinomorphic flowers are less advanced than zygomorphic, considering all that has been said of the influence of insect pollination in floral evolution. The suggestion that stipules are a primitive feature has already been made by Sinnott & Bailey (1914) and is well supported by the evidence of correlations. The establishment of integumentary vascular bundles as a primitive character is particularly interesting in view of the occurrence of vascular bundles in the integuments of Pteridosperms, Cycads, Gnetales and Conifers (except Pinaceae).

To the list of primitive characters might be added tentatively certain other characters which show some correlations. They should not, however, be used as yet for calculating the advancement index of families, largely because their correlations are based on very limited data. Thus, the facts of nodal anatomy (Sinnott & Bailey, 1914) are available for only 155 out of the 259 families of Dicotyledons, and while preliminary calculations suggest that these workers were correct in claiming that the tri-lacunar node is primitive, this conclusion cannot be taken as reliable until the remaining 104 families are included in the calculations. Similar remarks apply to the information concerning wood structure (Chalk, 1937). Preliminary calculations show slight negative correlations between 'advanced wood' and primitive characters, but since these calculations are based only on the 136 families mentioned by Chalk, wood structure has not been included among the characters used for calculating the advancement index of families. Information about pollination processes is very inadequate and is apparently completely lacking for about 100 families, but from what information is available in Müller (1883) and Knuth (1906) it appears that correlations exist between anemophily and both stipulate leaves and unisexual flowers. There is also a correlation between anemophily and haplochlamydy. It would appear, therefore, that anemophily is a primitive process and not, as Robertson (1904) suggested, one which has resulted from a process of reduction and degradation. But the final verdict on this question must await the accumulation of much more information. The basic primitive characters from which the advancement index is calculated have, accordingly, been limited to the twelve characters listed in Table 1.

THE ADVANCEMENT INDEX

In calculating an advancement index, an attempt is made to assess numerically the relative advancement of each of the 259 families of Dicotyledons. The method makes use of the information contained in the first twelve columns of Table 1, the relative number of advanced and primitive characters being determined for each family. In column 13 the advancement index is expressed as a fraction, and in column 14 as a percentage. The numerator of the fraction is an estimate of the number of advanced characters possessed by the family, and in making this estimate, each ' – ' counts one 'mark', while each ' ± ' counts half a mark. The denominator is the number of characters about which information is recorded for the family. Thus the Amaranthaceae score six marks for the six negative signs and one mark for the two ± signs. The numerator is therefore 7. The denominator is 11, since information is lacking on the subject of fusion of petals (in the absence of petals). The fraction $\frac{7}{11}$ gives the figure of 64% for the advancement index.

A possible criticism of this method of calculation is that each of the twelve characters is given equal weight in assessing primitiveness or advancement, whereas in fact, some are better indicators than others. As already stated, it is sometimes difficult to distinguish a primitive unisexual flower from one which is secondarily derived. This character is therefore less reliable as an indicator than the tree habit, for example. At some future date it is hoped to make some allowance for this and to attempt the distinction between primitive and derived characters. But for the present purpose, uncorrected estimates will suffice, so long as it is realized that the figures quoted for the advancement index cannot be taken in any exact sense, but give only a very rough estimate of relative advancement.

This is particularly the case in families which show great variations. The example already quoted is 'mixed' in respect of two characters (leaf arrangement and unisexuality). Now although unisexual flowers are rarely found in the Amaranthaceae, yet those species which do have unisexual flowers and which also have alternate leaves would have an advancement index of 55%. Conversely, those species with bisexual flowers and opposite leaves would have an advancement index of 73%. The figure of 64% is clearly, therefore, merely an average for the whole family, and there is considerable variation within the family, even though it is 'mixed' in respect of only two characters. The variations within a family which exhibits a greater number of ' ± ' signs is correspondingly greater. Thus, the Euphorbiaceae are mixed in respect of six characters, and although they are assigned an index of 25%, one species may approach 0% while other species might be as high as 50%. Some of the families which exhibit great range of floral and vegetative characters in this way are those which have been considered for splitting into smaller families. Thus, Leguminosae and Saxifragaceae both show variation in six characters, and both have now given place to a number of smaller families. It would seem that the Euphorbiaceae should be treated in a similar manner, and that unrelated genera which are at present brought together into this rather unnatural family should be transferred to a number of new families, some of which would, no doubt, be just as primitive as the Flacourtiaceae.

In the meantime, however, it will be of interest to arrange the families of Dicotyledons in order of their advancement index as calculated in Table 1, so that it can be seen at a glance which families are at the bottom of the scale and which are at the top.

Advancement index (%)	
8	Flacourtiaceae
12	Anonaceae, Magnoliaceae
18	Myristicaceae
25	Bixaceae, Bombacaceae, Euphorbiaceae, Malvaceae, Myrtaceae
29	Dilleniaceae, Nymphaeaceae, Rhizophoraceae, Sapindaceae
30	Fagaceae
33	Guttiferae, Leguminosae, Rhamnaceae, Salicaceae, Theaceae, Thymelaeaceae, Tiliaceae
36	Akaniaceae, Crossosomataceae, Lecythidaceae, Phytolaccaceae, Trochodendraceae
37	Aizoaceae, Dipterocarpaceae, Myricaceae, Platanaceae, Resedaceae, Stachyuraceae, Sterculiaceae
40	Achatocarpaceae, Betulaceae, Gyrostemonaceae
42	Begoniaceae, Caryocaraceae, Chlaenaceae, Cochlospermaceae, Didieraceae, Elaeocarpaceae, Eucryphiaceae, Marcgraviaceae, Monimiaceae, Ochnaceae, Punicaceae, Quiinaceae, Vitaceae
44	Myrothamnaceae
45	Aristolochiaceae, Buxaceae, Calycanthaceae, Cercidiphyllaceae, Eupomatiaceae, Lactoridaceae, Nepenthaceae, Pandaceae
46	Anacardiaceae, Canellaceae, Celastraceae, Combretaceae, Connaraceae, Cunoniaceae, Ebenaceae, Erythroxylaceae, Meliaceae, Nyssaceae, Passifloraceae, Ranunculaceae, Rosaceae, Sonneratiaceae, Staphyleaceae
50	Aextoxicaceae, Balanopsidaceae, Berberidaceae, Casuarinaceae, Cistaceae, Corynocarpaceae, Cucurbitaceae, Datiscaceae, Empetraceae, Eucommiaceae, Himantandraceae, Juglandaceae, Melianthaceae, Menispermaceae, Moraceae, Papaveraceae, Portulacaceae, Rhoipteleaceae, Simarubaceae, Ulmaceae, Violaceae

Advancement index (%)	
54	Burseraceae, Caricaceae, Hamamelidaceae, Hernandiaceae, Lardizabalaceae, Lauraceae, Lythraceae, Myrsinaceae, Olacaceae, Oxalidaceae, Polygalaceae, Saxifragaceae, Tamaricaceae, Tremandraceae, Turneraceae
55	Brunelliaceae, Daphniphyllaceae, Medusagynaceae, Piperaceae, Urticaceae
56	Leitneriaceae
58	Achariaceae, Actinidiaceae, Alangiaceae, Ancistrocladaceae, Araliaceae, Capparidaceae, Cneoraceae, Droseraceae, Hippocrateaceae, Icacinaceae, Linaceae, Melastomataceae, Moringaceae, Pentaphyllacaceae, Rutaceae, Sabiaceae, Salvadoraceae, Sapotaceae, Scytopetalaceae, Styracaceae, Zygophyllaceae
59	Basellaceae, Octoknemataceae, Polygonaceae
60	Cactaceae
62	Aceraceae, Aquifoliaceae, Caryophyllaceae, Coriariaceae, Dichapetalaceae, Elatinaceae, Frankeniaceae, Geraniaceae, Halorrhagaceae, Hippocastanaceae, Loasaceae, Penaeaceae, Saururaceae, Theophrastaceae
64	Amaranthaceae, Bretschneideraceae, Elaeagnaceae, Heteropyxidaceae, Hoplestigmataceae
65	Thelygonaceae
67	Clethraceae, Compositae, Cornaceae, Crassulaceae, Cyrillaceae, Fouquieraceae, Hydrocaryaceae, Malpighiaceae, Oleaceae, Oliniaceae, Onagraceae, Opiliaceae, Pittosporaceae, Plumbaginaceae, Rubiaceae, Sarraceniaceae, Symplocaceae, Tovariaceae, Trigoniaceae
68	Nyctaginaceae, Proteaceae, Strasburgeriaceae
70	Chloranthaceae, Julianaceae, Myzodendraceae, Rafflesiaceae
71	Diapensiaceae, Lissocarpaceae, Malesherbiaceae, Roridulaceae, Tropaeolaceae, Vochysiaceae
72	Podostemonaceae
73	Batidaceae, Byblidaceae, Cephalotaceae, Ceratophyllaceae, Diclidantheraceae, Geissolomataceae, Gomortegaceae, Santalaceae
75	Borraginaceae, Bruniaceae, Convolvulaceae, Cruciferae, Crypteroniaceae, Dysphaniaceae, Epacridaceae, Limnanthaceae, Loganiaceae, Myoporaceae, Stackhousiaceae, Stylidiaceae
77	Chenopodiaceae
78	Hydnoraceae
79	Apocynaceae, Balsaminaceae, Caprifoliaceae, Ericaceae, Hydrophyllaceae, Loranthaceae, Nolanaceae, Pirolaceae, Primulaceae, Umbelliferae
80	Garryaceae
82	Desfontainiaceae, Grubbiaceae
83	Asclepiadaceae, Brunoniaceae, Calyceraceae, Campanulaceae, Gentianaceae, Globulariaceae, Goodeniaceae, Hydrostachyaceae, Plantaginaceae, Solanaceae
85	Balanophoraceae, Cynomoriaceae
86	Lennoaceae
87	Callitrichaceae
88	Acanthaceae, Columelliaceae, Verbenaceae
92	Adoxaceae, Bignoniaceae, Gesneraceae, Lentibulariaceae, Orobanchaceae, Polemoniaceae, Scrophulariaceae
95	Hippuridaceae
96	Labiatae, Pedaliaceae, Valerianaceae
100	Dipsacaceae, Martyniaceae, Phrymaceae

It will at once be realized that among the families with the lowest advancement index are representatives from widely different orders. Thus, among the first fourteen families, four are placed in the Ranales, three in the Parietales, two each in the Malvales and the Myrtiflorae, and one each in the Geraniales, Sapindales and Fagales. Associated with these diverse relationships are diverse types of receptacle, for while three families have inferior ovaries and three have convex receptacles, the remainder have flat or saucer-shaped receptacles. Now, neither inferior nor superior ovaries seem to be correlated with any of the twelve primitive characters, and it seems probable that the convex receptacle is neither more nor less primitive than the inferior ovary, but that both began to evolve at a very early stage from some intermediate form with a flat or saucer-shaped receptacle. This is borne out to some extent at the other end of the scale, where among the fourteen most advanced families, three have inferior ovaries, eight have superior ovaries and two are 'mixed', while the Hippuridaceae are so reduced that neither sepals nor petals are present.

Another interesting point emerging from an examination of the upper end of the scale is that families with well-known parasitic or semi-parasitic representatives tend to have a high advancement index, e.g. Orobanchaceae, Scrophulariaceae, Lennoaceae, Balanophoraceae and Cynomoriaceae. The advanced mode of life of these families supports their high advancement index.

The catkin-bearing plants have an average advancement index of 47%, and while they range from the Fagaceae (30%) and Salicaceae (33%) to the Garryaceae (80%), most of them are nearer to 50%. It cannot be said, therefore, that catkin-bearing plants are primitive, as has sometimes been claimed, since some of them are relatively advanced. Neither can it be said that they are closely related and represent stages in a single line of evolution, for some have inferior ovaries and some superior. The group would appear to be an artificial one containing unrelated families, some advanced, like the Garryaceae, and representing a retrograde step from some bisexual ancestor, and some quite primitive, like the Salicaceae, with flowers which are fundamentally unisexual.

These examples show how the discovery of statistical correlations between floral and vegetative characters provides a new and objective approach to the problems of the primitive flower and evolution among the Dicotyledons, and provides the starting-point for the preparation of a more 'natural' classification. But much of what has been said concerning the Dicotyledons can be applied elsewhere. The definitions and the arguments based thereon are not peculiar to the Dicotyledons, but can be applied equally well to any large group of organisms, either animals or plants, and further study along the lines suggested in this paper may well lead to a more complete understanding of the course of evolution of all forms of life.

SUMMARY

By means of a statistical analysis of floral and vegetative characters possessed by the various families of Dicotyledons, it is shown that some tend to be correlated together and to occur together more often than they would if distributed at random throughout the families. Some of these characters also occur more often than would otherwise be expected in families known to have existed in geological deposits of Tertiary age. Such characters are therefore claimed as indicating primitiveness. The primitive Dicotyledon is pictured as a tree or a shrub, with alternate, glandular, stipulate leaves, with unisexual, actinomorphic, polypetalous flowers, with many stamens and many carpels, and with arillate seeds whose two integuments were vascularized. A rough estimate of relative advancement, in the form of an 'advancement index', shows that the most advanced families include Dipsacaceae, Labiatae, and Valerianaceae, while the most primitive families include Flacourtiaceae, Anonaceae, Magnoliaceae, Myristicaceae, Bixaceae, Euphorbiaceae, Malvaceae and Myrtaceae.

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REFERENCES

BLENK, P. (1884). Ueber die durchsichtigen Punkte in den Blättern. *Flora*, **42**, 371.

CHALK, L. (1937). The phylogenetic value of certain anatomical features of Dicotyledonous woods. *Ann. Bot., Lond.*, N.S. **1**, 409.

EDWARDS, W. N. (1931). *Fossilium catalogus*. II. Plantae. Pars 17: Dicotyledonae (Ligna). Berlin.

ENGLER, A. & DIELS, L. (1936). *Syllabus der Pflanzenfamilien*. Berlin.

ENGLER, A. & PRANTL, K. (1887–). *Die natürlichen Pflanzenfamilien*. Leipzig.

FISHER, R. A. (1936). *Statistical Methods for Research Workers*. Edinburgh and London.

HUTCHINSON, J. (1926). *Families of Flowering Plants. Dicotyledons*. London.

KNUTH, P. (1906). *Handbook of Flower Pollination*. Transl. J. R. Ainsworth Davis. Oxford.

LE MONNIER, G. (1872). Recherches sur la nervation de la graine. *Ann. Sci. Bot.* **15**, 233.

MÜLLER, H. (1883). *The Fertilisation of Flowers*. Transl. D'A. W. Thompson. London.

NETOLITZKY, F. (1926). Anatomie der Angiospermen-samen. In K. Linsbauer's *Handb. der Pflanzenanatomie*. II. Abt. 2. Teil. *Pteridophyten u. Anthophyten*, Band **10**.

REID, E. M. & CHANDLER, M. E. J. (1933). *The London Clay Flora*. Brit. Mus. (Nat. Hist.).

RENDLE, A. B. (1925). *The Classification of Flowering Plants. II. Dicotyledons*. Cambridge.

ROBERTSON, C. (1904). The structure of the flowers and the mode of pollination of the primitive Angiosperms. *Bot. Gaz.* **37**, 294.

SINNOTT, E. W. & BAILEY, I. W. (1914). Investigations on the phylogeny of the Angiosperms. I. Nodal anatomy and the morphology of stipules. *Amer. J. Bot.* **1**, 441.

SPORNE, K. R. (1948). Correlation and classification in Dicotyledons. *Proc. Linn. Soc. Lond.* 160th Session.

WILLIS, J. C. (1931). *Flowering Plants and Ferns*. Cambridge.

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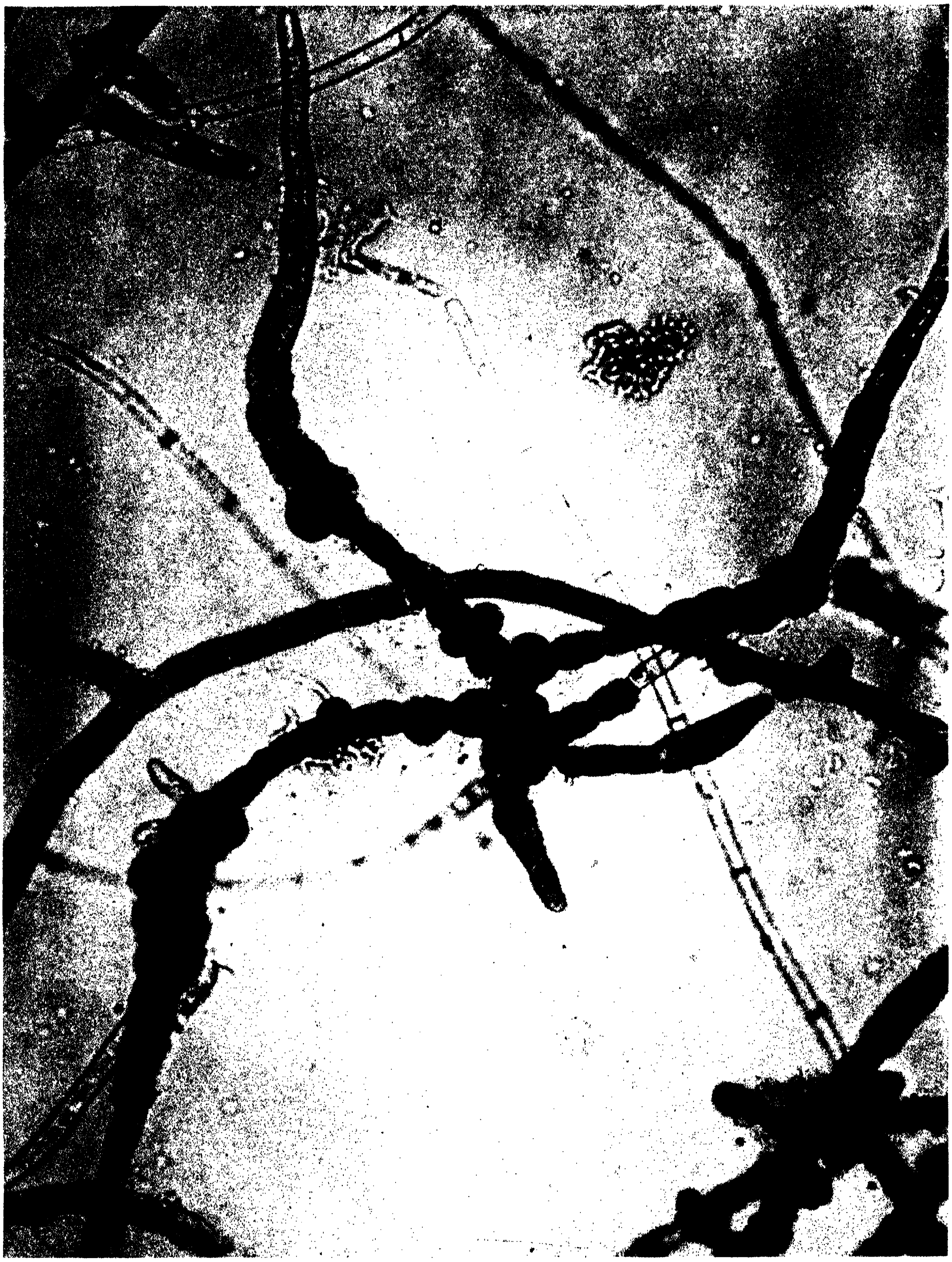

LERNER—A NOTE ON THE LIFE HISTORY OF *SPLACHNIDIUM RUGOSUM* L. (GREV.)

A NOTE ON THE LIFE HISTORY OF *SPLACHNIDIUM RUGOSUM* L. (GREV.)

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(With Plate 12)

In a recent letter to *Nature* Hopkins (1948) showed that the swarmers from the unilocular sporangia of *Splachnidium rugosum* L. (Grev.) do not fuse, but grow into microscopic filaments. During an investigation of the life history of this alga carried on by the writer in 1944, while at the University of Cape Town, similar observations were made. In culture the gametophytes were small (0·5–1 mm.), branched, uniserate filaments with one large dissected chromatophore per cell. The branches terminated in characteristic, long, colourless hairs, well shown in Pl. 12. When 6–8 weeks old some of the gametophytes produced plurilocular sporangia. These were somewhat irregular in outline, paler than the filament, tapering towards the apex and divided by longitudinal and cross-walls. The sporangia borne by some individuals had fewer cross-walls, the cells were therefore larger, suggesting anisogamy. Confirmation of this, and of the expected fusion of the zooids, was prevented by the degeneration without release of the spores. Thus the final proof that these sporangia actually contained gametes is still lacking.

The macroscopic plant, the sporophyte, bears at the apices of the branches a number of short hairs. These have been termed 'assimilators' by Fritsch (1945). In the living plant these are quite colourless and it seems, therefore, a mistake to call them this. Cells of the endophyte *Codiolum* were never found in specimens collected in Table Bay. These have very often (Fritsch, 1945) been figured as present at the apices of the branches and bases of the conceptacles.

If, as seems fairly certain, the plurilocular sporangia described here are really gametangia, the position of *Splachnidium* as a member of the Ectocarpales is confirmed. Further elucidation of its systematic relationships must, however, await knowledge of the developing sporophyte.

I should like to take the opportunity of thanking Dr M. R. Levyns of the Botany Department, Cape Town, for her continued help and encouragement.

REFERENCES

HOPKINS, R. J. (1948). Life History of *Splachnidium rugosum*. *Nature, Lond.*, **162**, p. 75.
FRITSCH, F. E. (1945). *The Structure and Reproduction of the Algae*, vol. II, p. 95.

EXPLANATION OF PLATE

Gametophytes with young plurilocular sporangia. Colourless hairs also shown.

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REVIEWS

Genera Filicum—the Genera of Ferns. By E. B. Copeland. Waltham, Mass.: Chronica Botanica, 1947.

The author, who spent many years in the Philippine Islands, where the Vascular Cryptogams are very numerous, has throughout his life devoted much of his time to the study of ferns, so that there can be no one with a better knowledge of pteridophytes in the field. This work is not his first essay at a phylogenetic classification of the Filicales, but the culmination of several attempts. The chief obstacle to a satisfactory classification of recent ferns has always been the difficulty of dealing with the huge and unwieldy family of the Polypodiaceae, which includes about 7500 species. This difficulty was appreciated by Bower (*The Ferns*, III, 1928) who endeavoured to trace phyletic lines, but never set out his conclusions as a formal classification with described and defined families and genera. Copeland (1947) and Holttum (1947) have now independently attempted to frame classifications more in line with a phylogenetic basis. The great genera *Dryopteris* and *Polypodium*, which were recognized as polyphyletic by Bower, are here subdivided as by Christensen, and the segregates sometimes referred to different families. Thus *Dryopteris* is restricted to the male fern and presumably its allies such as *D. dilatata*, *D. spinulosa* and *D. aemula* by both authors, and placed in the family Aspidiaceae by Copeland, and in Dennstaedtiaceae subfam. Dryopteroideae by Holttum.

Unfortunately, though there is a key (on p. 18) the differences between the families are not very clearly set out. In a previous paper (Copeland, 1929, p. 47) Copeland stated that there were three phyletic series in the Polypodiaceae, traceable to the Cyatheaceae, Dicksoniaceae and Matoniaceae respectively, but if these groups were to be treated as families, the resulting families would be undefinable. The families now proposed, or rather adopted from the works of older authors, are largely based on facies rather than fitted into a scheme of classification made up of selected characters, as in most artificial classifications, and this makes definition difficult; indeed Copeland (p. 100) describes his own definition of Aspidiaceae as 'useless'. This is also true of some of the accepted families of dicotyledons.

Copeland's Vittariaceae are an exceptionally clear-cut group, as is shown by the characters given on p. 227. Of these the most important is considered to be the elongate prothallus. Bower's family Plagiogyriaceae is taken up, but his suggested affinities are rejected. The Davalliaceae, characterized by articulate stipes (except *Nephrolepis*), normally indusiate sori, free veins, sori terminal on the veins, etc., seems a very natural group. The Polypodiaceae have also an articulate stipes, but are strictly exindusiate, with free or reticulate veins and sori terminal, lateral, or forming coenosori. The Pteridaceae, Aspidiaceae, Blechnaceae, and Aspleniaceae, in which the stipes is not articulate, are less satisfactory, but the first named are often characterized by the presence of hairs in place of scales, marginal sori, and tetrahedral spores. The other three families have bilateral spores.

Most of the Aspleniaceae are characterized by the presence of two vascular bundles at the base of the stipes, which unite to form a more or less X-shaped strand. The Blechnaceae and Aspidiaceae seem undefinable, except that an epispore is almost always present in the former and commonly wanting in the latter.

If this classification is applied to the native British genera, *Pteridium*, *Cryptogramma*, *Anogramma*, and *Adiantum* fall under Pteridaceae; *Woodsia*, *Polystichum*, *Dryopteris*, *Thelypteris* (Copeland's *Lastrea* which includes *Gymnocarpium*), *Cystopteris*, and *Athyrium* under Aspidiaceae; *Blechnum* (including *Lomaria*), Blechnaceae; *Asplenium* (including *Phyllitis*), and *Ceterach* represent the Aspleniaceae; while *Polypodium* remains the sole representative of the Polypodiaceae.

This classification seems as satisfactory as any yet proposed, and attention may be directed to some of the apparently exceptional or anomalous genera, which do not quite fit into the places assigned to them. With so many segregates of the large genera, it may be convenient to regard some of them as subgenera pending more monographic treatment, while some of Copeland's families might be better placed as subfamilies until more definite distinctions are found. The work is well produced and printed and merits the careful study of all interested in the taxonomy of the Pteridophyta.

A. H. G. ALSTON

REFERENCES

Holttum, R. E. (1947). A revised classification of Leptosporangiate ferns. *J. Linn. Soc. Bot.* **53**, pp. 123–58.

Copeland, E. B. (1929). The oriental genera of Polypodiaceae. *Univ. Calif. Publ. Bot.* **16**.

Botany of the Canadian Eastern Arctic. Part II. Thallophyta and Bryophyta. Compiled and edited by NICHOLAS POLUNIN. 9¾ × 6½ in., pp. v+573; 18 plates, 5 text-figures and folding map. Canada Department of Mines and Resources; National Museum of Canada Bull. no. 97 (Biological series no. 26). 1947. Price $1.

This bulletin contains the following seven contributions: Algae (other than those dealt with by other contributors) by Roy M. Whelden; Marine Phytoplankton by Gunnar Seidenfaden; Fresh-water Diatomeae by Robert Ross; Fungi by David H. Linder; Lichenes by Bernt Lynge; Musci by William C. Steere; Hepaticae by Nicholas Polunin. To these seven papers Prof. Polunin contributes an introduction stating the general scope of the work, the conventions adopted, etc., and a summary showing in tabular form the known distribution of the majority of the species in the region covered by the flora, i.e. the Canadian Arctic north of the 60th parallel and east of long. 95° W., with the exclusion of the Sverdrup Islands, and certain parts of the mainland.

The scope of the papers varies considerably. Whelden and Linder list only the species gathered by Polunin in this region. Ross gives records from collections by other travellers which are preserved at the British Museum, but he has examined only a part of the available material. The remaining contributions gather together all available records for the region. Each contribution has an introduction giving in chronological sequence the history of the previous collecting of the group in the region, and a full bibliography which would enable previous records to be located even when they are not given in full. Seidenfaden and Steere both summarize some of the salient features of the floras in concluding sections. The bulk of the space is devoted, however, to the systematic list giving for each species a statement of its general distribution, and its known distribution in the Canadian Eastern Arctic. There are additional notes on the species which vary greatly in scope and character; sometimes these are merely critical notes on systematy; sometimes they are of a more general nature. Linder's notes on the Fungi appear to be particularly valuable, since they include a number of keys to genera, many notes on the biology of the species and diagnoses. There are a number of new species and/or varieties described in all groups except the Bryophytes (it would have been useful to have an index to these new forms). The plates illustrate algae, fresh-water diatoms and fungi.

It is obvious that these critical and scholarly accounts of the cryptogamic flora of an important but much-neglected part of the Arctic will be of great value in providing a starting-point for future students of the vegetation of the region, and they will also be a valuable mine of information on the geographical distribution of species within the Arctic. To the non-specialist their appeal is necessarily much slighter, though even he may find some points of interest. No new species of bryophyte are described, and the proportion of new forms appears to increase from the lichens through the Algae to the Fungi. In the bryophytes and lichens the records of earlier collectors make a substantial proportion of the whole; with the algae and fungi it is clear that Polunin's own collections constitute the foundation of our knowledge, and the omission of earlier records is of no very great importance. Polunin's own gatherings of algae on his 1936 expedition provided 'more than four times as many species as have been recorded from our area by any one previous authority'. These facts are interesting commentaries on the state of our systematic knowledge of the various groups. Steere comments on the fact that collecting of bryophytes in the Canadian Arctic has always been done casually by non-specialists, and that a specialist would doubtless find very much more; if this is true of the bryophytes, how much more must it be true of the algae and fungi?

This flora represents one of the few attempts to bring together into compact and readily accessible form the whole of the known flora of an Arctic region; it provides us with a kind of grand 'Biological Spectrum'. It is of course obvious that the numbers of species for the lesser collected groups in particular are minima, yet since Polunin appears to have collected everything with equal enthusiasm even they are probably not without comparative value. The numbers of the various groups recorded are as follows:

Phanerogams	297	Cyanophyceae	108
Musci	304	Fresh-water diatoms	192
Hepaticae	75	Desmids	184
Lichenes	275	Other Chlorophyceae	63
Fungi	79	Phaeophyceae	12
Marine plankton (Diatoms)	79	Rhodophyceae	9
Marine Plankton (Peridineae)	39	Miscellaneous	6

Steere comments on the way in which the Arctic mosses greatly exceed the flowering plants in numbers, and often seem to become the dominant group of plants. Lichens are obviously about as rich in species, and it is probable that the diatoms and desmids would equal or exceed these in numbers if they were better known. Whelden and Ross both comment on the richness of the material which they examined; a single gathering from a tundra-pool sometimes had forty or fifty species of diatoms and another forty or fifty species of other algae. Of the fungi eighteen are the larger Basidiomycetes; the remainder are parasitic or saprophytic stem or leaf fungi, or aquatic.

E. W. JONES

A Manual of the Timbers of the World. By ALEXANDER L. HOWARD. 9 × 6½ in. Pp. xxiv + 751, with upwards of 100 illustrations in the text. London: Macmillan and Co., Ltd. 1948. 52*s.* 6*d.*

The third edition of Howard's well-known work gives some indication of the large number of woods which have become known since the first World War, for the space devoted to descriptions of the world's timbers is more than double that required in the first edition, which appeared in 1920.

As in previous editions, the woods are described in alphabetical order, the title name being, where practicable, the common or trade name. Since the author had a lifetime's experience of timber from the trade aspect, the articles on the better known commercial timbers like Walnut, Oak and Mahogany are, as might be expected, authoritative and contain a wealth of information which is not available elsewhere. Thus under African Mahogany an heroic attempt has been made to reduce chaos to order: it would take a rash scientist indeed to attempt a differentiation of the commercial varieties of this timber—Assinee, Bonamba, Benin, Cape Lopez and the rest, but it can, apparently, be done with fair accuracy by the expert merchant, using such data as texture, colour, sizes of logs, degree of bruising of the logs and so on.

Some of the less well-known woods, of course, receive scant treatment, but in some instances this brevity can scarcely be attributed to lack of information. Thus, *Piptadenia africana*, appearing under one of its less familiar names instead of Dahoma, which is not even mentioned, is dismissed in some half dozen lines, while *Gossweilerodendron balsamiferum* (Agba) and *Terminalia superba* (Afara) claim little more attention. The explanation of such parsimonious treatment is probably to be found in the bibliography: no claim is made that the list of books is exhaustive, but it will occasion some surprise that such standard works as those on Empire and Home-Grown Woods of the Forest Products Research Laboratory at Princes Risborough, the Oxford series on Empire trees and timbers, Dadswell and others on Australian Woods and Moll and Janssonius on those of Java, to mention but a few, are omitted. It should have been possible to give more comprehensive information with such books available.

Moreover, it is difficult to avoid the feeling that the author's excursion into wood structure is not a happy one: his concern here has been purely with identification, but it would be idle to contend that a description of one or two features of a wood is sufficient for this purpose, except in special instances. For example, the description for a possible *Chamaecyparis* reads 'The lines of concentric layers are marked by darker golden brown shading to a lighter colour', while the diagnosis for almond runs 'The medullary rays are well marked, and the pores, except in the pore zone, are very small'. It is difficult not to feel that space occupied by such descriptions—and in aggregate it is not small—might have been so much more profitably used for those aspects of the subject on which the author was an authority.

There is an appendix which classifies the world's timbers by their country of origin, a useful addition to the book, but not free from mistakes and misleading statements. It is not questioned, for example, that Alder and Hawthorn occur in Africa, but no one would expect to obtain wood of these species from such a source, while Dhup is surely a name applied to certain Asiatic and not African woods. Here also Robinia appears, under the extraordinary trinomial of *Acacia Robinia pseudacacia*, and as an Indian wood.

Lest these adverse comments suggest that the book is one which need not be consulted, it must be emphasized that it is little short of indispensable to the specialist. The author's experience of timber, acquired not only in Britain, but from visits to a number of timber-producing countries, must have been unique and it is to this commercial aspect that the book owes its value.

Nor would the review be complete without reference, first to the illustrations, which have been selected with care and reproduced with skill, and also to the book itself: it is a pleasure to handle a book printed on good paper at the present time.

F. W. JANE

British Flowering Plants. Evolution and Classification of Families and Genera with Notes on their Distribution. By JOHN HUTCHINSON. $5\frac{1}{2} \times 8\frac{1}{2}$ in. Pp. viii+374, with 174 black and white illustrations and 22 colour plates by the author. London: P. R. Gawthorn Ltd. 1948. Price 25*s*.

'"Taxonomy without phylogeny may be likened to bones without flesh." This book is intended to supply for the student of British botany a little "flesh", and thus add to the interest of our fascinating, though somewhat meagre flora.' It seems doubtful, however, whether this book will appeal to students of the British flora who lack an initial interest in phylogenetic problems, or will satisfy those who wish to weigh the evidence for Dr Hutchinson's views. In an introductory chapter, chiefly historical and apologetic in content, the division of Dicotyledons into two main lines, one fundamentally and predominantly woody, the other herbaceous, is adopted more emphatically than in earlier works, while the conventional division into Archichlamydeae and Metachlamydeae is completely discarded. There follow the 'principles of classification adopted in this new system'. These principles, twenty-two in number, are stated without discussion or presentation of evidence, although they include several about which there will be considerable difference of opinion. Such are: 'As a rule simple leaves are older than compound leaves', 'The solitary flower is more primitive than the inflorescence', 'Contorted (1), imbricate (2) and valvate (3) floral parts (sepals and petals) have been evolved in this sequence', and 'Flowers with petals are generally of a more primitive type than those without petals'. Dr Hutchinson's familiar and interesting but by no means wholly acceptable views of the phylogenetic interrelations of the orders and families of British seed-plants are then presented in a series of tables. The remainder of the book deals with the families in turn, providing keys for distinguishing their British genera, supplying some items of information about them and indicating the author's views as to the paths of evolution both between and within the families, but still without supporting evidence. The family Gramineae, the account of which was supplied by Mr C. E. Hubbard, is treated at much greater length than any other, with sections on the evolution of the spikelet, fruits, vegetative and anatomical characters and cytology, as well as keys to tribes and genera and fairly lengthy discussions of their content and phylogeny. This chapter reveals what a fascinating and valuable book, or series of books, Dr Hutchinson might have provided for the serious student had he aimed at a more satisfyingly detailed consideration of each family in the British flora.

The exposition is marred by such lapses as 'Having become tired of fighting the herbs it (*Clematis vitalba*) has climbed the hedges where it is perfectly happy and contented', and there are a few misstatements and printer's errors. The numerous line drawings, chiefly by the author, are clear and informative, but rather stiff. There are also twenty-two colour plates by the author, variable in merit but, as a whole, difficult to justify in such a book as this. A. R. CLAPHAM

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(1) *Diseases of Cereals.* Pp. x+69, with 25 figures. (2) *Diseases of Potatoes, Sugar Beet and Legumes.* Pp. viii+86, with 27 figures. By W. A. R. DILLON WESTON; illustrated by Ann Murray. $8\frac{1}{2} \times 5\frac{1}{2}$ in. Longmans, Green and Co. Paper, price 4*s*. each.

These two booklets (a companion volume on the diseases and pests of vegetables is forthcoming) are based on a series of articles contributed to *Agriculture*, the monthly Journal of the Ministry of Agriculture and Fisheries. A special feature is the illustrations, mostly full-page life-cycle diagrams which convey a great deal of information in a simple and effective manner. The quotation from *The Horse-Hoing Husbandry* of Jethro Tull, prefixed to the first volume, which so aptly justifies this method of presentation, is a measure of the author's skill in the selection and arrangement of his subject matter.

The 'Cereals' volume deals first with fungus diseases and their spread in general, and with the practice of seed disinfection. Thereafter it covers in succession the smut fungi, leaf-stripe fungi, ergot, mildew, the rusts, root and stem rot fungi, and other miscellaneous ailments and abnormalities. Finally, the diseases, with their symptoms and control measures, are summarized in tabular form under their several hosts.

The second volume opens with two striking chapters on the potato in plenty and in famine, leading to the treatment in the third chapter of the major diseases, blight, leaf roll, and dry rot. Gappy crops, scab and wart disease, and the two potato eelworm diseases are then dealt with, leaving the last four chapters respectively for the diseases of sugar beet and mangolds, beans, peas, and clover.

Though somewhat highly priced for their size and binding (paper and printing are excellent), these books deserve a very wide sale: they are particularly commended to agricultural students.

T. E. T. BOND

An Introduction to Plant Anatomy. By A. J. EAMES and L. H. MACDANIELS. 9 × 6 in. Pp. xviii + 428 with 186 figures in the text. New York and London: McGraw-Hill. 2nd edition, 1947.

There can be no text-book on plant anatomy half so useful to the University student of Botany (and his teachers) as that written by two colleagues of Cornell University, Professors A. J. Eames and L. H. MacDaniels. Since the first publication in 1925 their book has become indispensable, and it is with pleasure that we greet a second edition, and the prospect of easier access to it. How well the work has stood the test of time! Because so largely based upon the personal working experience of the two authors, and because of a thoroughly critical initial approach, there has been need for very little excision.

The changes are mostly by way of additions to particular sections of morphology or anatomy, wherein knowledge has recently expanded. Thus there is included a brief treatment of new information of the anatomy of the cell wall; a fuller treatment of meristems is now made, and this includes a wise and cautious introduction to the *Tunica-corpus* theory of the stem apex. Leaf ontogeny receives more attention, and flower, fruit and seed are dealt with somewhat more fully than before. Extended treatment of the phloem reflects the recently increased understanding of that tissue.

The authors have resisted suggestions to expand the work further, and have admirably succeeded in retaining the popular character and scope proper to the University text-book.

Excellent new illustrations supplement the old ones, particularly of course in the newly expanded sections. Where so many good new illustrations have been provided, may we suggest that it would help the beginning student of wood anatomy to see illustrated 'aggregate' and 'compound' rays, which are such distinctive features of certain timbers, but which are so hard to visualize from written descriptions? Similarly, the ontogeny of the stomata, and the commoner groupings of accessory cells around them would be worth showing.

H. GODWIN

Genetics in Swedish Forestry Practice. By BERTIL LINDQUIST. Stockholm, Svenska skogsvärdsföreningens förlog; Waltham, Mass., U.S.A.: Chronica Botanica Co.

This is an English translation of Dr Lindquist's book, which first appeared in Swedish in 1946 and was reviewed in vol. 47 of this *Journal* (p. 148). There has been little or no change in content.

ED.

Svalöf 1886–1946: *History and Present Problems.* Edited by A. AKERMAN, O. TEDIN and K. FRÖIER. English Technical Editor, R. O. WHYTE. Roy. 8vo. Pp. v + 389. Lund: Carl Bloms Boktryckeri A.-B. 1948. 30*s.*

This book has been compiled by members of the Swedish Seed Association to commemorate the sixtieth anniversary of the founding of the Association in 1886 at the village of Svalöf near the southern tip of Sweden, for the primary purpose of breeding improved crop plants. The book, which is intended to encourage 'the international collaboration of scientific plant breeders', comprises a series of some twenty-two articles in English by various authors, each dealing with a particular branch of the work. An introductory chapter on the Swedish Seed Association, its foundation, organization, development and activities is given by W. E. Ljung, who was secretary and treasurer of the Association for over thirty years. In addition to the main plant-breeding institute at Svalöf, a number of subsidiary centres have been established in other parts of Sweden, to deal with varieties of crop plants adapted to local conditions of climate and soil. These conditions are described by K. Andersson, and the work of the branch stations is described by A. Akerman and E. Akerberg. Three papers are devoted to a general discussion of fundamental problems of plant breeding, namely, the methods of breeding self-fertilized plants (A. Akerman and J. MacKey) and of cross-fertilized plants (G. Nilsson-Leissner) and the effects of inbreeding in herbage plants

(G. Julen). Breeding methods and the results obtained for individual Swedish crop plants are described, each species by a specialist in the work. Recent work on artificially-induced polyploidy is discussed by A. Levan and by A. Müntzing, and mutation work is described by A. Gustafsson and J. MacKey. The book concludes with an account by J. Lindberg of the cereal and chemical laboratories, and a description by K. Fröier of the publications of the Association.

The various articles are generally of a high standard of accuracy, but a few minor points of criticism may be noted. It is suggested (pp. 343, 351) that the absence of *albina* mutants in *Pisum sativum* and *Lupinus luteus* is due to a 'genotypical directing of the mutation process', since such mutants occur in *Trifolium pratense* and other species. However, no mention is made of the equally plausible hypothesis that mutation occurs at random, and that the *albina* mutants may be inviable in some species. On p. 52, the word 'homozygozation', though clear in meaning, is fortunately not in general use in genetical literature. Some confused sentences (end of first paragraph on p. 58 and near bottom of p. 62) require correction. The word 'self-fertility', 8 lines from the bottom of p. 214, should read 'self-sterility'.

Among many observations of considerable interest to geneticists, the following may be mentioned: the genetic simulation of a virus disease of potatoes (pp. 140–1); the induction by X-ray treatment of an advantageous chlorophyll mutant in flax (pp. 191–2, 343); and a remarkable heterosis phenomenon following irradiation with low and medium doses of X-rays (p. 348).

An important aspect of the Swedish plant-breeding work is the widespread use of natural selection as an aid to crop improvement. The success achieved by this method has stimulated attempts to intensify the effects of natural selection by subjecting crop plants to infection with common diseases, to artificial freezing and to drought (p. 209). This book will undoubtedly prove a useful volume of reference for plant breeders, and sets an example which, if followed by other plant-breeding institutes, should simplify and accelerate the spread and interchange of advances in plant breeding. One of the most valuable features of the book is the full bibliography given at the end of each paper, and in particular a complete list (pp. 318–23) of publications by members of the cyto-genetic department since its foundation in 1931. H. L. K. WHITEHOUSE

NOVEMBER 1949 VOL. 48, NO. 3

RACIAL DIVERGENCES IN *PRUNELLA VULGARIS* IN RELATION TO HABITAT AND CLIMATE

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(With Plates 13–16, and 1 figure in the text)

1. INTRODUCTION, MATERIALS AND METHODS

My preliminary work on the variability and life-forms of *Prunella vulgaris* (Böcher, 1940) describes the main features of the variation of the species from a morphological point of view. By means of a study of thirteen different cultures at that time I found the same main types as those which can now be set up after studying 138 cultures from different places. The main types were characterized by their life-form, it being possible to distinguish between hemicryptophytes, which only reach flowering in the second season of cultivation, hemicryptophytes, which flower in the first season, biennial hemicryptophytes and therophytes (annuals). In a later work (Böcher, 1945*a*), importance was attached to an examination of certain important physiological differences that are correlated with the differences in life-forms, viz. differences in the influence of winter conditions (cold and day length) on shoot development and floral initiation. In the present work I shall endeavour, on the basis of extensive cultivation experiments, to elucidate the racial structure of the species and to examine the relations between the races or the various heritable features and the habitat and climate.

Most of the cultures I shall refer to were made with plants from various natural localities. From a number of colleges abroad I received seed samples, and I myself collected seeds in Iceland, the Faroe Islands, Norway and Denmark. Unfortunately, I was able to obtain only two seed samples of wild *Prunella* from southern Europe, so that the material from there is somewhat scanty; on the other hand, it has been supplemented by a number of seed samples from botanic gardens in southern Europe. For these samples I am unable to record the original natural locality of the plants; but it is permissible to assume that at any rate most were in south Europe; this assumption is borne out in no unmistakable manner by the cultures themselves, for the resulting plants on the whole were different from those originating from more northerly regions. The material from western Europe is also scanty, owing to the fact that during the war I was out of contact with colleagues in England.

For practical reasons it was impossible to sow the seed samples all at once. Nor could all cultures be undertaken in the same experimental garden. This means that the measurements of the various plants are only comparable within the same experiment; direct comparisons can only be made on plants started simultaneously and grown at the same place. Had differences in size been the principal criteria for *P. vulgaris*, this method of cultivation would have made comparisons impossible within the material taken as a whole; as it is, size is of secondary importance, because the chief difference lies in habit-type and development-type, both of which are influenced only very slightly by the small difference in conditions between experimental gardens in the same locality or by different dates of

Table 1. *Synopsis of material*

Locality	No.	Culture no.	Origin	Habitat	Collector	Exp. no.	Plate or Fig. no.
North-west and western Europe	1	333	Iceland, Reykjavik	Dry riverside	G. Guðjonsson	V	Pl. 15
	2	885	Geysir	Meadow at rivulet	T.W.B.	IX	
	3	886	Reykjavik	Grassland in valley	T.W.B.	IX	
	4*a*	887	Reykjavik	Grassland near road	T.W.B.	IX	
	4*b*	888	Reykjavik	Grassland near road	T.W.B.	IX	
	5	889	Tingvellir	Herb slope	T.W.B.	IX	
	6	99	Faroes, Thorshavn	Montane meadow	T.W.B.	I, V	Text-fig. 1
	7	68	Thorshavn	Lowland pasture	F. H. Møller	III	Pl. 16, fig. 1
	8	69	Kirkebø	Alpine vegetation	F. H. Møller	III–IV	Pl. 16, fig. 1
	9	890	Thorshavn	Roadside	T.W.B.	IX	
	10	70	Norway, Bykle	Subalpine grassland	T.W.B.	III–IV	
	11	71	Skudesnæshavn	Meadow	T.W.B.	IV	
	12	72	Haugesund	Meadow	T.W.B.	III–IV	
	13	211	Eire, Dublin	Botanical Gardens	—	I	
	14	227	England	Natural habitat	E. M. Marsden-Jones	II	
Denmark, excluding the Faroes and Bornholm	15	257	North Jutland, Gerum Hede	Meadow in valley	T.W.B.	III	
	16	258	Frederikshavn	Meadow	T.W.B.	III	
	17	259	Pikerbakken	Pasture	T.W.B.	III	
	18	314	Østerild	Moist meadow	T.W.B.	V	
	19	480	Bjerget	Moist meadow	T.W.B.	VII	
	20	486	Bulbjerg	West slope at the sea	T.W.B.	VII	
	21	492	Gasbjerg	Chalk grassland	T.W.B.	VII	
	22	100	Risum Enge	Open soil in grazed meadow	T.W.B.	I	
	23	557	Ll. Blaakilde	Chalk grassland	T.W.B.	VIII	
	24	558	Næsby Dale	West slope at the Limfjord	T.W.B.	VIII	Pl. 16, fig. 2
	25	560	Djørup	Dry cultivated grassland	T.W.B.	VIII	
	26	319	South Jutland, Borris	Dry cultivated grassland	T.W.B.	V	
	27	318	Ring Sø	Moist meadow	T.W.B.	V	Text-fig. 1
	28	367	Tønning	Dry pasture	T.W.B.	V	
	29	417	Gudensø	Wet meadow	T.W.B.	VI	
	30	435	Kulsø	Meadow	T.W.B.	VI	
	31	434	Ø. Nykirke	Rather dry cultivated grassland	T.W.B.	VI	
	32	599	Ø. Nykirke	Roadside, not dry	T.W.B.	VIII	
	33	598	Lerbæk Ml.	Pasture, rather dry	T.W.B.	VIII	
	34	597	Rugballegd.	Grazed meadow	T.W.B.	VIII	
	35	607	Lerbæk Skov	*Fraxinus* wood on slope	T.W.B.	VIII	
	36	611	Grejsdalen	Beechwood on slope	T.W.B.	VIII	

37	428		Randbøldal	Wet meadow	T.W.B.	VI	
38	430		Vittrup	Rather dry cultivated grassland	T.W.B.	VI	
39	321		Bindeballe	Dry pasture (acid soil)	T.W.B.	V	
40	260		Fuglsang Skov	Wood	T.W.B.	III	
41	415		Jørgensgd. Skov	Wood	T.W.B.	VI	
42	429		Aabenraa	Road in wood	T.W.B.	VI	
43	431		Agnsø	Wood	T.W.B.	VI	
44	414		Hostrup Sø	Lakeshore	T.W.B.	VI	
45	243	Danish Islands,	Læsø	Meadow at Hotemmen	T.W.B.	III–VI	
46	436		Arreskov Sø	Meadow	T.W.B.	VI	
47	316		Refsnæs	Rather dry grassland	T.W.B.	V	
48	331		Refsnæs	*Juncus inflexus* society	T.W.B.	V	
49	596		Hovvig	Meadow near saltmarsh	T.W.B.	VIII	Text-fig. 1
50	604		Skredbjerg	Grassy slope	T.W.B.	VIII	
51	498		Tudsenæs	Pasture	T.W.B.	VII	
52	497		Bogenæs	Grassy northern slope	T.W.B.	VII	
53	603		Egholm Skov	Road in wood, not shady	T.W.B.	VIII	
54	475		Allindelille	Chalky grassland	T.W.B.	VII	
55	476		Gyrstinge	Lakeshore, dry marly soil	T.W.B.	VII	
56	323		Gilbjerghoved	Grassy northern slope	T.W.B.	V	Text-fig. 1
57	322		Arresø	Moist meadow	T.W.B.	V	
58	294		Arresø	Moist meadow	T.W.B.	V	Pl. 15
59	535		Gribskov	Roadside	T.W.B.	VII	
60	609		Gurre Sø	Meadow in *Alnus* wood	T.W.B.	VIII	
61	532		Espergærde	Pasture	T.W.B.	VII	
62	610		Ermelunden	Wood, somewhat shaded	T.W.B.	VIII	
63	295		Fortunen	Dry roadside	T.W.B.	V	
64	330		Hareskov	Wood	T.W.B.	V	
65	329		Virum	Dry roadside	T.W.B.	V	
66	328		Virum	Rather dry cultivated grassland	T.W.B.	V	Pl. 13
67	327		Rudersdal	Rye field on clay	T.W.B.	V	
68	326		Frederikslund	Roadside near wood	T.W.B.	V	
69	244		Gels Skov	Wood	T.W.B.	IV	Text-fig. 1
70	245		Skodsborg	Wood	T.W.B.	V	
71	899		Holte	Reed swamp at pond	T.W.B.	IX	
72	437		Søndersø	*Alnus glutinosa* wood	T.W.B.	VI	
73	438		Jonstrup Vang	Reed swamp, shaded	T.W.B.	VI	Pl. 13
74	432		Kongelunden	Road in moist wood	T.W.B.	VI	
75	433		Kongelunden	Meadow	T.W.B.	VI	
76	552		Møns Klint	Bank of brooklet	T.W.B.	VII	

Table 1 (*continued*)

Locality	No.	Culture no.	Origin	Habitat	Collector	Exp. no.	Plate or Fig. no.
The Baltic, Sweden, north-east Europe	77	575	Denmark, Bornholm	Rather dry meadow	V. Mikkelsen	VIII	
	78	300	Sweden, Øland	Meadow near the sea	Th. Arwidsson	V	
	79	299	Øland	Alvar vegetation	Th. Arwidsson	See p. 305	Text-fig. 1
	80	302	Rydbo St.	Wood	Th. Arwidsson	V	
	81	297	Stocksund	The harbour	Th. Arwidsson	V	Text-fig. 1
	82	440	Busjön, Värml.	Moist pasture, 130 m.a.s.	Hård av Segerstad	VI	
	83	401	Gotland	*Sesleria-Festuca ovina-Campylium stellatum* meadow	B. Pettersson	VIII	
	84	600	Gotland	*Pinus* wood on rocky ground	B. Pettersson	VIII	
	85	409	Finland, Tyrväntö	Lakeshore, 82 m.a.s.	A. Vaarama	VI	Pl. 13
	86	231	Latvia, U.S.S.R., Riga	Natural habitat	—	II	
	87	232	U.S.S.R., Wilno	Botanical Gardens	—	III	
	88	531	Kenjärvi	Natural habitat	—	VII–VIII	
	89	220	Leningrad	Botanical Gardens	—	I	
	90	219	Moscow	Botanical Gardens	—	I	
	91	210	Penza	Botanical Gardens	—	I	
East France, central Europe	92	208	France, Nancy	Natural habitat	—	I	
	93	411	Luxemburg, Drauffeld	Meadow	P. Michaelis	VI	Pl. 13
	94	412	Eifel, Dann	Meadow	P. Michaelis	VI	
	95	413	Westfalen, Minden	Meadow	P. Michaelis	VI	
	96	410	Buckow at Berlin	Meadow	P. Michaelis	VI	
	97	296	Müncheberg at Berlin	Rather dry meadow	P. Michaelis	V	
	98	303	Wald-Sieversdorf at Berlin	Road in dry pinewood	P. Michaelis	V	
	99	375	Poland, Poznan	Botanical Gardens	—	V	
	100	311	Thüringia	Rather dry grassland	H. Meusel	V	Pl. 15
	101	419	Schlesia, north-east of Lissa	Grassy slope at lake (Lindensee)	P. Michaelis	VI	
	102	422	Frauenhain	Moist meadow at a pool	P. Michaelis	VI	
	103	421	south of Glatz	Moist meadow	P. Michaelis	VI	
	104	248	Hohenheim	Botanical Gardens	—	III	
	105	312	Maria Eich at Munich	Rather dry grassland	H. Meusel	V	
	106	423	Czechoslovakia, Sbirow	Meadow (*Colchicum*)	P. Michaelis	VI	
	107	426	Sudomirsch	Roadside	P. Michaelis	VI	
	108	418	Adamsthal	Margin of wood	P. Michaelis	VI	
	109	420	Eisgrub	Grassy slope	P. Michaelis	VI	
	110	247	Poland, Krakov	Botanical Gardens	—	III	
South-east Europe	111	427	Austria, Laxenburg	Meadow in the park	P. Michaelis	VI	
	112	253	Hungary, Budapest	Botanical Gardens	—	III	
	113	230	Szeged	Natural habitat	—	II	
	114	212	Rumania, Cluj	Botanical Gardens	—	I	

Alps	115	216	Switzerland, Zürich	Botanical Gardens	—	I	
	116	251	France, Grenoble	Botanical Gardens	—	III	
	117	213	Austria, Graz	Botanical Gardens	—	I	
	118	306	Innsbruck	Semi-natural manured vegetation, 600 m.a.s.	H. Gams	V	
	119	305	Planötzen	Pasture, 700 m.a.s.	H. Gams	V	
	120	307	Arzler Alpe	Pasture, 1100 m.a.s.	H. Gams	V	Text-fig. 1
	121	308	Guttal	Pasture, 1930 m.a.s.	H. Gams	V	Pl. 13
	122	402	Heiligenblut	Montane vegetation	P. Michaelis	VI	
	123	396	Arnoldstein	Gravelly soil	P. Michaelis	VI	
	124	397	Nötsch	Roadside at a luxuriant meadow, 600 m.a.s.	P. Michaelis	VI–VII	
Southern Europe	125	249	Portugal, Lisbon	Botanical Gardens	—	III, VI	Pl. 13
	126	207	Coimbra	Natural vegetation	A. Fernandes	I	
	127	268	Coimbra	Natural vegetation	A. Fernandes	III	Text-fig. 1
	128	301	Quinta do Prado	Granite soil, 950 m.a.s.	A. Fernandes	V	
	129	293	Spain, Madrid	Botanical Gardens	—	VI	
	130	376	France, Montpellier	Botanical Gardens	—	V	
	131	209	Italy, Ventimiglia	Botanical Gardens	—	I	
	132	268	Mortola	Botanical Gardens	—	III	
	133	252	Trieste	Botanical Gardens	—	III	
	134	198	Modena	Botanical Gardens	—	I	
	135	324	Urbino	Botanical Gardens	—	VI	Pl. 13
	136	250	Rome	Botanical Gardens	—	III	
	137	194	Canda, Toronto	Botanical Gardens	—	I	
	138	315	Jutland, Hovsør, Thy	Dry grassy slope	T.W.B.	See p. 311	
	139	606	Tinnet Krat	Dry cultivated grassland	T.W.B.		
	140	602	Danish Islands, Krogenlund	*Alnus* wood	T.W.B.		
	141	614	Hammerbakke	Grassy slope	T.W.B.		
	142	601	Sweden, Gotland	*Carex panicea* meadow	B. Pettersson		
	143	214	Canada, Montreal	Botanical Gardens	—		Text-fig. 1
Samples of *Prunella grandiflora* or *P. grandiflora* × *vulgaris*							
	144	594	Denmark, Kyndby	Natural vegetation (see Böcher, 1945*b*, Table III and Plate V)	T.W.B.	See Table 10	Text-fig. 1
	145	309	Austria, Höttinger, Graben	*Pinus silvestris-Erica carnea* wood, 700 m.a.s.	H. Gams		
	146	310	Planötzen, Innsbruck	700 m.a.s.	H. Gams		Text-fig. 1
	147	398	Villacher Alpe	Alpine meadow, 1900 m.a.s.	P. Michaelis		Text-fig. 1

sowing. I have had some opportunities both of comparing the same material in the same year in two different gardens, and in two different years in the same garden. In one case alone I was able to observe a small difference with regard to development-type (see p. 300), but never any difference in habit-type.

With some of the samples I varied the experimental conditions for the purpose of studying phenotypic variation, the plants, after a single repotting, usually being left in pots of 14 cm. diameter.

The pot-culture experiments and most of the field cultures were made in the University Botanical Gardens with their experimental fields. One small experiment was made at the experimental garden of the Genetic Laboratory of the Royal Veterinary and Agricultural College, Copenhagen. The four experimental gardens utilized will be referred to as A (Albertslund, Vridsløselille), B (Botanical Gardens), F (Nørre Fælled, Experimental Garden) and L (Royal Veterinary and Agricultural College, Lyngby).

Here I should like to thank Prof. Knud Jessen, Director of the Botanical Gardens, and Prof. C. A. Jørgensen, Royal Veterinary and Agricultural College, for placing these gardens at my disposal. My thanks are also due to colleagues abroad who helped in collecting seed samples, and finally to the Carlsberg Foundation and the Rask-Ørsted Foundation for financial support in the handling and publication of the material.

2. LIFE-FORM: HABIT-TYPE AND DEVELOPMENT-TYPE

As the main differences in *P. vulgaris* are biophysiological in nature and become manifest only during development from seed to flowering, transplant experiments are of little value. One must sow seeds and then follow the plant for at least two years, preferably more, in order to study these differences. The purely morphological differences can of course be studied in series of transplants, but these, too, become most distinct in material started as seedlings.

Habit-type means the habit of a plant after flowering has commenced. Plants such as *Plantago maritima* or *P. lanceolata* are easily characterized, because they do not possess the power of vegetative propagation and have an exceedingly simple structure (cf. Gregor's works and Böcher, 1943). Compared with these plants, *Prunella* is more difficult because consideration must be given to (1) the direction of the spike-bearing shoots, and (2) the power of vegetative spread. As with *Plantago* the *direction of the spike-bearing shoots* may be designated by the names *erecta*, *adscendens* and *procumbens*; each of these three types constitutes a range within the total variation of the species. The *adscendens* type is easily the most common; *procumbens* is very uncommon and found in only one sample (no. 93); *erecta* is also fairly rare. In *erecta* most of the stems are erect, whereas in *adscendens* the majority are ascending.

The very different capacity for vegetative propagation in the different samples is illustrated in Böcher (1940, Fig. 3). Some types have epigeal rooting stolons which may be branched, some send out stolons which end in rosettes, whereas others creep but little because new wintering rosettes are formed at or near the base of the plant (cf. Pl. 14). However important these biological-morphological differences may be, they are not particularly suitable for characterizing the appearance of the plants. A better method is to adopt a broader evaluation of the power of vegetative reproduction by means of three other morphological types, which may suitably be called *caespitosa*, *pulvinata* and *repens*. Allocating the plants to these types is easy. *Caespitosa* is a type with little or no capacity for creeping, the dense leaf rosette appearing in the first season of cultivation being preserved for a long period. The plant consists of a basal, hemispherical, leafy tussock from which the long spike-bearing stems emanate. In the *pulvinata* type the leafy region is flattened out to a more or less broad cushion from which the flowering stems emanate. Finally, there is the *repens* type, which forms flat, widespread carpets by reason of its great capacity for creeping.

By combining the two systems of morphological types it is possible to express the appearance of a plant, its habit-type, fairly well by two names, e.g. *pulvinata-adscendens* (very common), *caespitosa-erecta*, *repens-adscendens*, etc.

In many cases the spike-bearing shoots rise from the entire plant, but just as often there is a difference between the central and the peripheral region, the most vigorous shoots coming from the margin of the tussock or cushion. In some cases this is because a large wintering leaf-rosette is retained in the middle; in others the cause seems to be that the soil in the centre gradually becomes less suitable than round the periphery. It is not unusual for the plant to die away in the middle and form a ring. The same has been observed in *Prunella grandiflora*, though here the plant does not die in the centre, this part being occupied by a very luxuriant cushion of leaves.

Development-type. This expression covers the manner in which the plant develops from seedling into spike-bearing unit and onwards, until it eventually dies. As long ago as 1856 Irmisch formed the opinion that *P. vulgaris* when cultivated can flower in the first or second season. After flowering it may die or continue by means of basal lateral shoots. Hoffmann (1887) and Sylvén (1906) also mention first- and second-year flowering *P. vulgaris*. My studies (Böcher, 1940, 1945*a*) have also disclosed marked differences as regards duration of life, first flowering and manner of wintering.

The great majority of my *P. vulgaris* samples are hemicryptophytes. Only very few proved to be annuals when cultivated in the open. Experiments on cultivating some of these indoors in winter (Böcher, 1945*a*) make it extremely probable that in warmer climates the latter are also hemicryptophytes, as they continue to live whenever they escape the winter cold (cf. p. 302). Accordingly, there can hardly be any reason for a sharp distinction between annual and perennial forms, the transition being fairly smooth; it is important, however, to be able to emphasize the differences in *duration of life* observed in culture under equal or practically equal conditions. For this purpose I have elected to use Roman numerals corresponding to the period of years in which the plants remain alive. *P. vulgaris* types living one, two, three, four and more years were found; these are indicated by the symbols I, II, III, IV and ♃ (♃ means perennial).

It is often found that those plants which flower earliest also die earliest. This is by no means the rule, however, and therefore we must also have an expression, independent of that for the duration of life, for *the rate at which the plants develop from seedlings to flowering units*. Plants have been found which in culture flower 0, 1 and 2 years after sowing; this is indicated by Arabic numerals 1, 2 and 3, signifying the year of cultivation in which flowering begins. Type 3 is very rare (see nos. 83–84 mentioned on p. 293).

Some Prunellas flower early, others late, and others again at an intermediate time, neither early nor late. For plants which succeed in flowering in the first season, the first inflorescence is always fairly late in appearing. Consequently, in order to make comparisons we must examine the *earliness* in the second season. The three types, early, intermediate and late, are expressed by the letters *e*, *i* and *l*.

Just as the habit-types are expressed by combinations of two terms, development-types may be expressed by terms for rate of development, earliness and life duration, e.g. 1 *e* I–II (flowers first year, early, annual-biennial) or 2 *l* ♃ (flowers second year, late, perennial).

3. CULTIVATION EXPERIMENTS

All the cultures were started from sowings in April. The seedlings were planted out in the garden in June. Of each number, ten or a few more were usually planted, at least five others being kept in pots for other purposes. A total of nine experiments were carried out (I–IX). The plants were measured at the close of the season in which flowering began, and also often at the end of one or more of the following seasons. The measurements were of the highest shoot, of the width at the broadest place, and the spike length of the longest spike. Thus it was always the maximal values that were taken. The mean value of these maxima and the limits of variation are shown in Table 2, which also gives the ratio of mean height to mean breadth (*B*/*H*), a ratio which provides a supplement to the estimated habit-types.

Experiments I and II. These were begun in 1938 and 1939 respectively, in garden B. Exp. I has already been described in detail (Böcher, 1940, p. 3). Exp. II was a direct continuation of I and comprised only three types from natural stations in England (no. 14), Latvia (no. 86) and Hungary (no. 113). These three cultures differed rather considerably. No. 113 had a curious structure not seen on any other; its spikes were very close together in cymes or head-like inflorescences.

Experiments III and IV. Both were started in 1940 in garden B (no. III) and L (no. IV). Four specimens were cultivated in both gardens. On the differences between the plants grown in the two places see p. 300. On the whole the *caespitosa* type dominated, as in Exp. IX. This was due particularly to the many northern specimens cultivated in these experiments, although samples from southern regions (Trieste and Budapest) had also to be placed to the same group. The Trieste plants were unusually large, a feature observable already in the rosettes, which were more domed and with lighter coloured leaves than the rosettes from Norway and the Faroe Islands. As summer advanced, the Trieste plants retained the leaves on the spike-bearing shoots, but put out no new shoots, or only weak ones, on the ground; all the plants died during the winter. In contrast to this sample, no. 8 from the Faroe mountains quickly shed the leaves on the spike-bearing shoots, but in most cases retained the rosettes, and they survived the winter well.

Experiment V. In this large experiment, which was begun in 1941 in garden F, there were unusually many interesting samples, and the biological differences were especially prominent. Two extremes will be seen in Pl. 1. One, no. 66 (culture no. 328) came from a rather dry pasture in Denmark, the other, no. 121 (culture no. 308) from a mountain pasture in Kärnten, 1930 m. above sea-level. The alpine flowered long before its neighbour and was entirely withered and leafless at the top when the photo was taken at the beginning of September 1942. At this time no. 66 was still flowering and the leaves had not even started to wither. Other similar differences, and differences in mortality, appear from Pl. 3.

An annoying surprise was caused by no. 128. The seed, collected by Prof. A. Fernandes, came from a montane region in Portugal. Compared with the other sowings the seed germinated slowly and late. Twenty seedlings were planted out in the field and twenty more were potted in a frame with a rather high temperature. In no case did the plants flower in the first season. In the field in September 1941 there were twenty very vigorous, leafy plants which, judging from all experience, would be capable of surviving the winter, but they all died in the severe cold that followed, whereas all the other samples which had not flowered the first season did well. The twenty potted plants fared just as badly. However, the leafy plants were not genuine rosette plants, but consisted of a system of more or less erect branches, on which the leaves formed a hemisphere. At a certain time in the development the annuals nos. 126 and 127 from Coimbra in Portugal were also leafy without being rosette plants. Afterwards the branches elongated and set spikes. As far as no. 128 was concerned this did not occur in the first season, at any rate not in our climate. Possibly this is a short-day plant. The other Portuguese, nos. 126 and 127, are day-length indifferent (Böcher, 1945*a*).

Experiment VI. This, too, was a large experiment, started in 1942 in garden F. Here again there were extreme contrasts. The most striking was the difference between no. 93 from Luxemburg and no. 73 from Denmark. The latter is an *erecta* type, the former a *procumbens* type so extreme that its mean height was right down at 6·6 cm., whereas its breadth was 27·5 cm. The Danish *erecta* type reached the extremely low *B/H* value of 0·8, whereas the Luxemburg plant had a *B/H* value of 4·2, representing the other extreme; the plants were practically prostrate. In this experiment there was another surprise. No. 85 from the banks of a Finnish lake (seeds collected by Dr A. Vaarama) had formed twenty vigorous rosettes by the autumn of 1942 (Pl. 13). Nevertheless, they all died in the winter 1942–3, whereas the other rosette plants, including many from Denmark and Germany, managed very well. That winter was not very cold. In this case the demise of the plants is rather incomprehensible. The only explanation I can see lies perhaps in the changeable character of the winter in Denmark. In Finland it is fairly certain that the plant is covered with snow rather constantly and therefore protected in winter. In Denmark it is sometimes exposed to black frost, which perhaps it cannot withstand.

Experiments VII and VIII. These experiments, commenced in 1943 and 1944 respectively in garden F, were less comprehensive and were intended for rounding off the material with types not examined before. Two samples from Gotland (seeds from Dr B. Pettersson) were especially interesting (nos. 83 and 84). They grew at an unusually slow rate and were much inclined to remain in the rosette state. In no. 84 this was so marked that only one of eight plants succeeded in putting forth a single spike-bearing shoot in the second season. This puts it in a separate class, for it seems to require a 2-year period in the rosette stage.

Experiment IX. A small experiment with plants from Iceland, the Faroes and Denmark was started in 1947 in garden A. All the plants were second-year flowering, and most of them of the *caespitosa* type. All the samples were perennial, the rosettes being very vigorous at the end of the second and third summers.

The many measurements and observations summarized in Table 2 provide an impression of the variation of the species. Even when compared with other species which I have cultivated, the variation seems to be very considerable. Not many of the samples are exactly alike. As a rule the variation within one sample was moderate or slight; in particular, habit-type and development-type did not vary much. When the variation within one sample was particularly great and thus gave cause to fear that the seed came from a very heterogeneous population, the plants were not measured. In other cases no measurements were taken because the number of individuals was unusually low and because, as regards the measured characters, the plants presented nothing special in the way of deviation.

A survey of the frequency of the various morphological and physiological characters that form habit-type and development-type will be found in Table 3. As direct comparisons can be made only between plants cultivated simultaneously, each experiment is accounted for separately, except III and IV, which were carried out in the same year in two different gardens. Observations of samples cultivated in both gardens in these two experiments showed no differences as regards habit and development, and therefore it was considered justifiable to put them together. The results of the various experiments reveal wide mutual differences, due principally to the samples in the various experiments having come from localities so very different. For instance, it is very distinctly observed that Exps. III, IV and IX contain very many samples from northern regions, and that Exp. VII includes a large number from wet localities. Of secondary importance are differences in environment. It may be mentioned here that the high frequency of biennials in Exps. III and IV is probably due to the fact that after the second year, when prolific flowering usually weakens the plants, there followed an uncommonly hard winter which killed plants otherwise capable of thriving. In order to avoid too many categories the transitional cases like *caespitosa-pulvinata* have consistently been grouped together with *caespitosa*, and samples with almost equal numbers of first- and second-year flowering individuals are

Exp. no.	No.	Culture no.	No. of plants	Height (*H*) in cm. mean and range	Breadth (*B*) in cm. mean and range	*B*/*H*	Spike length in cm. mean and range	Habit-type	Development-type
I	6	99	15	15·7 (13–21)	23·8 (20–25)	1·5	4·0 (3·0–5·0)	*caesp. adsc.*	2 *e* IV–♃
	22	100	15	41·7 (37–46)	*c.* 40–50		*c.* 4	*caesp. adsc.-erecta*	2 *i* II
	13	211	10	42·8 (38–49)	54·0 (50–55)	1·3	4·4 (3·8–5·2)	*pulv. adsc.*	2 *l* IV–♃
	92	208	10	28·5 (26–32)	56·0 (40–62)	1·9	4·9 (3·5–6·0)	*pulv.-rep. adsc.*	1 *i* I–(V)
	115	216	10	28·2 (13–33)	53·0 (45–60)	1·9	3·4 (2·2–4·5)	*pulv. adsc.*	(1) 2 *i*–*l* ♃
	117	213	10	25·3 (23–28)	37·1 (35–40)	1·5	4·5 (4·0–5·5)	*caesp.-pulv. adsc.*	1 *i* II
	134	198	10	20·1 (17–24)	21·2 (19–24)	1·1	3·5 (3·2–3·6)	*caesp. adsc.*	2 *e* III–IV
	131	209	10	17·2 (12–24)	25·0 (22–29)	1·5	2·6 (2·0–3·0)	*pulv. adsc.*	(1) 2 *e* II–IV
	114	212	5	Not measured		Not measured		*pulv. adsc.*	2 *i* ♃
	89	220	10	26·4 (20–34)	42·0 (38–50)	1·6	3·4 (2·5–3·5)	*pulv. adsc.*	2 *e* ♃
	90	219	10	28·2 (26–30)	52·0 (36–60)	1·8	4·2 (3·5–5·0)	*pulv. adsc.*	2 *i* ♃
	91	210	6	Not measured		Not measured		*pulv.-rep. adsc.*	1 (2) *i* ♃
	137	194	10	43·0 (36–48)	67·0 (55–80)	1·6	7·4 (5·5–9·5)	*pulv. adsc.-erecta*	2 *l* ♃
II	14	227	15	42·2 (34–50)	73·9 (56–83)	1·7	4·7 (3·5–6·0)	*pulv. adsc.*	(1) 2 *i* II–IV
	86	231	10	33·0 (26–45)	57·0 (47–70)	1·7	4·8 (4·4–5·0)	*pulv. adsc.*	2 *l* II–(III)
	113	230	10	34·8 (20–48)	45·5 (29–62)	1·3	4·1 (3·5–5·0)	*caesp.-pulv. adsc.*	2 *e* II
III	7	68	10	20·1 (19–23)	32·3 (27–37)	1·6	3·8 (3·6–4·0)	*caesp. adsc.*	2 *e*–*i* ♃
	8	69	10	24·3 (22–27)	31·8 (30–33)	1·3	3·7 (3·6–4·0)	*caesp. adsc.*	2 *e* (II)♃
	12	72	10	16·9 (8–25)	30·6 (24–36)	1·8	3·5 (3·0–4·1)	*caesp.-pulv. adsc.*	2 *e*–*i* II–III
	45	243	10	20·5 (12–26)	32·3 (25–40)	1·6	3·5 (2·5–4·2)	*pulv. adsc.*	2 *l* II–IV
	17	259	10	19·3 (14–23)	40·9 (33–50)	2·1	2·8 (2·5–3·2)	*repens adsc.*	2 *l* II–IV
	16	258	10	22·3 (18–26)	47·6 (44–56)	2·2	3·1 (2·5–3·8)	*rep.-pulv. adsc.*	2 *i* II
	15	257	10	19·4 (14–26)	40·6 (29–46)	2·1	2·9 (2·3–3·5)	*rep.-pulv. adsc.*	2 *l* IV–♃
	40	260	10	29·6 (22–36)	39·6 (34–45)	1·3	3·2 (2·6–3·6)	*caesp. erecta*	2 *l* II
	104	248	10	15·8 (11–19)	54·3 (40–70)	3·4	3·4 (2·8–3·6)	*repens adsc.*	2 *l* ♃
	110	247	10	Not measured		Not measured		*pulv. adsc.*	2 *e*–*i* ♃
	87	232	10	38·1 (26–45)	51·7 (39–68)	1·4	4·5 (4·0–5·5)	*caesp. adsc.*	1 *l* II
	112	253	10	35·4 (32–38)	43·7 (38–50)	1·2	3·9 (3·6–4·5)	*caesp. adsc.*	2 *e* II
	133	252	10	35·3 (26–40)	46·5 (44–50)	1·3	4·8 (4·4–5·2)	*caesp. erecta*	(1) 2 *i* II
	132	286	10	30·1 (23–42)	41·9 (30–50)	1·4	4·8 (3·5–6·0)	*caesp. adsc.*	1 *l* (I) II
	136	250	10	22·0 (14–27)	45·2 (38–52)	2·1	4·7 (4·2–5·2)	*caesp.-pulv. adsc.*	1–(2) *i* I—(III)
	127	268	20	28·8 (25–33)	20·2 (14–28)	0·7	3·9 (3·0–5·0)	*caesp. adsc.-erecta*	1 *i* I (II)
	125	249	15	30·8 (23–42)	49·6 (42–57)	1·6	4·6 (4·0–5·5)	*caesp. adsc.-erecta*	1 *i* II–(♃)
	116	251	8	41·2 (37–46)	71·6 (68–75)	1·7	6·3 (5·5–7·0)	*caesp. adsc.*	2 *i* II
IV	8	69	10	12·8 (9–17)	19·5 (15–25)	1·5	2·5 (1·8–3·3)	*caesp. adsc.*	2 *e* (II) ♃
	12	72	10	21·5 (16–28)	40·1 (34–44)	1·9	3·5 (2·8–4·2)	*caesp. adsc.*	2 *e*–*i* II–III
	11	71	11	17·3 (14–23)	35·8 (32–42)	2·1	3·1 (2·8–3·8)	*caesp. adsc.*	2 *e*–*i* ♃
	10	70	8	18·9 (13–29)	25·3 (18–34)	1·3	2·6 (2·0–3·2)	*caesp. adsc.*	2 *e* II–III
	45	243	9	19·4 (17–22)	35·1 (26–40)	1·8	2·8 (2·2–3·7)	*pulv. adsc.*	2 *l* II–IV
	69	244	10	30·2 (26–34)	44·6 (39–57)	1·5	3·3 (3·2–3·5)	*caesp. adsc.-erecta*	2 *e*–*i* (II)–♃
V	6	99	10	17·7 (14–22)	29·0 (20–38)	1·6	4·8 (4·0–5·5)	*caesp. adsc.*	2 *e* IV–♃
	1	333	9	17·5 (15–19)	40·5 (35–50)	2·3	4·0 (3·8–4·3)	*caesp.-pulv. adsc.*	2 *e* II–IV
	18	314	10	21·1 (12–30)	52·7 (35–66)	2·5	5·3 (4·0–7·0)	*pulv. adsc.*	2 *l* ♃
	28	367	9	17·0 (10–23)	43·0 (32–58)	2·5	3·3 (2·5–4·0)	*pulv. adsc.*	1 *i* III–♃
	27	318	9	Not measured		Not measured		*pulv. adsc.*	2 *i*–*l* II–IV
	39	321	12	14·7 (8–25)	38·0 (26–67)	2·6	4·1 (3·0–5·0)	*pulv. adsc.*	1 *e* I–II (♃)

	56	323	11	26·5 (19–37)	67·5 (48–84)	2·5	5·1 (4·5–6·0)	*pulv. adsc.*	2 *l* ♃
	57	322	12	36·0 (19–47)	68·3 (52–90)	1·9	4·1 (4·0–4·5)	*pulv. adsc.*	2 *l* ♃
	58	294	10	22·1 (16–32)	43·4 (34–56)	2·0	4·0 (3·0–5·0)	*pulv. adsc.*	2 *l* ♃
	48	331	10	24·6 (16–29)	46·7 (35–54)	1·9	4·1 (3·5–5·0)	*pulv. adsc.*	2 *l* ♃
	47	316	10	25·8 (22–33)	61·0 (52–70)	2·4	4·5 (4·0–5·0)	*caesp.-pulv. adsc.*	1 *e* I–II (♃)
	64	330	10	36·4 (32–42)	56·7 (42–55)	1·6	5·3 (4·5–6·0)	*caesp. erecta*	2 *i* II–IV
	70	245	10	13·4 (9–21)	35·4 (26–47)	2·6	3·7 (2·5–5·0)	*repens adsc.*	1 *e* IV–♃
	68	326	10	30·7 (25–34)	72·0 (64–78)	2·3	4·6 (4·0–5·5)	*pulv. adsc.*	2 *l* ♃
	67	327	10	24·8 (19–30)	63·0 (54–72)	2·5	4·5 (4·0–6·0)	*pulv. adsc.*	2 *i* ♃
	66	328	10	20·8 (12–29)	58·4 (48–72)	2·8	3·7 (3·0–4·0)	*repens-pulv. adsc.*	1 (2) *l* ♃
	65	329	12	27·0 (18–35)	67·6 (38–85)	2·5	4·7 (4·0–5·0)	*pulv. adsc.*	1 (2) *i* I–(III)
	63	295	10	Not measured		Not measured		*pulv. adsc.*	1 *e* I–II
	81	297	11	13·4 (8–22)	39·6 (30–50)	2·9	4·0 (3·0–5·5)	*pulv. adsc.*	1 *e* ♃
	80	302	10	18·1 (10–25)	45·2 (30–53)	2·5	4·7 (3·5–6·0)	*pulv. adsc.*	2 *i* ♃
	78	300	10	22·4 (14–27)	40·9 (23–56)	1·8	3·8 (3·5–4·5)	*caesp. adsc.*	2 *e* III–♃
	97	296	10	Not measured		Not measured		*pulv. adsc.*	1 *l* (I)♃
	98	303	10	28·2 (12–46)	62·1 (42–80)	2·2	4·0 (3·5–4·5)	*pulv. adsc.*	1–2 *i* (II)♃
	100	311	13	16·2 (14–22)	36·2 (28–53)	2·2	3·6 (3·0–4·0)	*pulv. adsc.*	1 (2) *i* I–(III)
	105	312	12	26·0 (22–30)	60·0 (46–72)	2·3	4·1 (3·5–4·5)	*pulv. adsc.*	1 (2) *e–i* I–(III)
	118	306	10	29·1 (23–36)	55·1 (41–66)	1·9	4·6 (4·0–5·0)	*pulv. adsc.*	1–2 *l* (I)♃
	119	305	11	19·6 (12–28)	52·4 (40–62)	2·7	3·8 (3·5–4·5)	*caesp.-pulv. adsc.*	2 *l* ♃
	120	307	11	8·3 (4–12)	21·0 (13–26)	2·5	2·8 (2·0–3·2)	*caesp. (adsc.)*	2 *i* ♃
	121	308	10	22·4 (13–29)	37·1 (18–49)	1·7	3·9 (3·0–5·0)	*caesp. (adsc.)*	2 *e* ♃
	99	375	10	Not measured		Not measured		*pulv. adsc.*	1 *i* I–(II)
	130	376	10	26·0 (16–37)	49·7 (32–62)	1·9	4·0 (3·5–4·5)	*pulv. adsc.*	2 *i* (II)♃
	128	301	20	Not measured		Not measured		*caesp. erecta*	2 (cp. p. 293)
VI	85	409	20	Not measured		Not measured		?	2 (cp. p. 294)
	82	440	10	11·4 (7–15)	21·0 (12–29)	1·8	1·6 (0·7–2·2)	*caesp. adsc.*	2 *e–i* II–III
	26	319	10	19·0 (14–25)	31·6 (19–37)	1·7	3·5 (3·0–4·0)	*caesp. adsc.*	2 *e* II–♃
	30	435	18	12·3 (6–19)	28·8 (17–42)	2·3	2·0 (0·8–3·3)	*pulv. adsc.*	2 *i–l* ♃
	29	417	10	20·8 (16–28)	59·6 (45–73)	2·9	2·4 (2·0–3·0)	*pulv. adsc.*	2 *e–i* ♃
	31	434	12	11·7 (7–15)	27·6 (18–54)	2·4	1·7 (0·8–4·0)	*caesp.-pulv. adsc.*	2 *e* II–IV
	38	430	10	18·0 (11–26)	46·5 (28–62)	2·5	2·2 (1·2–3·3)	*caesp.-pulv. adsc.*	2 *i* ♃
	37	428	20	10·8 (7–17)	24·9 (14–42)	2·3	1·5 (0·7–3·1)	*caesp. (adsc.)*	2 *e* ♃
	42	429	15	22·3 (18–27)	34·1 (30–43)	1·5	4·1 (3·2–5·0)	*pulv. erecta*	2 *e* ♃
	43	431	10	19·0 (10–26)	32·3 (20–40)	1·7	2·8 (1·8–4·5)	*caesp. adsc.*	2 *e* ♃
	41	415	10	14·2 (12–17)	34·6 (28–47)	2·4	3·6 (2·8–6·0)	*caesp.-pulv. adsc.*	2 *i* II–(♃)
	44	414	5	Not measured		Not measured		*caesp.-pulv. adsc.*	2 *i* ♃
	46	436	10	11·2 (9–14)	41·2 (38–44)	3·7	2·6 (1·9–3·4)	*repens adsc.*	2 *i–l* ♃
	72	437	10	22·8 (12–30)	23·5 (16–28)	1·0	3·0 (2·3–3·6)	*caesp. adsc.-erecta*	2 *e–i* II
	73	438	10	41·3 (32–58)	32·0 (24–42)	0·8	3·3 (2·7–4·0)	*caesp. erecta*	2 *i–l* (II)–♃
	74	432	10	30·2 (22–37)	49·7 (39–62)	1·6	5·2 (4·0–8·5)	*pulv. erecta*	2 *i–l* ♃
	75	433	11	36·2 (19–48)	70·3 (57–90)	1·9	4·6 (3·3–5·2)	*pulv. adsc.*	2 *l* ♃
	93	411	11	6·6 (4– 8)	27·5 (19–41)	4·2	1·1 (0·7–1·5)	*repens procumbens*	2 *i* (III)–♃
	94	412	14	11·7 (7–19)	40·1 (26–50)	3·4	2·2 (1·0–3·5)	*repens-pulv. adsc.*	2 *i* III–♃
	95	413	13	13·5 (10–20)	45·9 (26–69)	3·3	2·7 (1·1–4·0)	*repens-pulv. adsc.*	2 *l* IV–♃
	96	410	17	27·3 (14–39)	63·5 (32–75)	2·3	3·3 (2·0–4·0)	*repens-pulv. adsc.*	2 *i* IV–♃
	101	419	15	20·3 (15–25)	48·2 (33–67)	2·4	3·4 (3·0–4·0)	*pulv. adsc.*	2 *i* ♃
	102	422	16	18·4 (12–31)	48·9 (37–76)	2·7	3·3 (2·6–5·0)	*pulv. adsc.*	2 *l* IV–♃
	103	421	10	19·3 (9–24)	45·7 (37–60)	2·4	3·1 (2·0–4·0)	*pulv. adsc.*	2 *l* ♃
	106	423	10	14·3 (9–20)	42·0 (32–48)	3·0	2·0 (1·5–2·5)	*pulv. adsc.*	2 *i–l* II–IV
	107	426	10	14·1 (9–30)	43·0 (32–81)	3·3	2·1 (1·4–3·5)	*caesp.-pulv. adsc.*	2 *l* III–IV
	108	418	5						

Exp. no.	No.	Culture no.	No. of plants	Height (*H*) in cm. mean and range	Breadth (*B*) in cm. mean and range	*B*/*H*	Spike length in cm. mean and range	Habit-type	Development-type
VI (*cont.*)	109	420	12	12·4 (7–17)	35·1 (22–46)	2·8	1·8 (1·0–2·7)	*repens-pulv. adsc.*	2 *l* (II) ♃
	111	427	10	35·2 (24–46)	61·4 (50–68)	1·7	4·7 (3·2–6·0)	*pulv. adsc.*	2 *i* ♃
	122	402	12	10·1 (6–16)	21·8 (16–26)	2·2	2·1 (0·8–3·8)	*caesp. adsc.-erecta*	2 *e* III–♃
	124*a*	397	9	18·3 (15–20)	34·7 (27–40)	1·9	3·6 (3·2–4·2)	*caesp. adsc.*	2 *e* ♃
	124*b*	397	17	12·9 (7–22)	27·3 (18–37)	2·1	2·1 (1·6–2·8)	*caesp. adsc.*	2 *e* ♃
	123	396	9	17·0 (11–22)	34·3 (27–50)	2·0	2·9 (2·0–3·4)	*caesp. adsc.*	2 *e–i* III–♃
	135	324	10	9·1 (6–14)	19·7 (17–24)	2·2	2·0 (1·6–3·3)	*caesp. adsc.*	1–2 *e* I–II
	129	293	10	29·3 (18–41)	55·3 (41–75)	1·9	4·2 (3·0–5·5)	*pulv. adsc.*	2 *i* ♃
	125	249	17	27·1 (19–41)	44·1 (30–65)	1·6	5·5 (3·5–7·0)	*caesp. adsc.-erecta*	1 *i* II (♃)
VII	21	492	10	20·4 (18–23)	33·6 (22–40)	1·6	4·0 (3·0–4·4)	*caesp. adsc.*	(1) 2 *e* II
	19	480	10	15·6 (12–22)	30·3 (23–40)	2·0	4·3 (3·0–6·0)	*caesp.-pulv. adsc.*	1–2 *l* II–IV
	20	486	10	12·8 (9–17)	31·2 (22–39)	2·4	4·3 (3·5–5·0)	*caesp. adsc.*	2 *e* II–III
	61	532	7	15·0 (13–17)	58·6 (40–76)	3·9	4·5 (4·0–5·0)	*repens-pulv. adsc.*	2 *i* ♃
	59	535	22	* (16–42)	* (25–60)		* (2·5–5·0)	*pulv. adsc.-erecta*	1–2 *i* (I)–IV
	55	476	10	30·5 (20–38)	46·8 (35–58)	1·5	4·3 (3·0–6·0)	*pulv. adsc.-erecta*	1–2 *i* ♃
	54	475	10	14·5 (10–22)	40·2 (35–46)	2·8	2·9 (2·0–4·0)	*pulv. adsc.*	2 *i* (*l*) ♃
	52	497	7	26·1 (23–28)	65·3 (62–69)	2·5	4·7 (4·5–5·0)	*pulv. adsc.*	2 *i* II–IV
	51	498	5	Not measured		Not measured		*repens-pulv. adsc.*	(1) 2 *i* ♃
	76	552	10	11·5 (7–15)	25·3 (17–38)	2·2	3·6 (2·0–5·0)	*caesp. adsc.*	2 *i* II
	88	531	17	29·0 (23–43)	49·9 (30–60)	1·7	4·8 (3·5–6·0)	*caesp. adsc.*	2 *e* ♃
	124*c*	397	10	20·6 (15–26)	34·2 (30–39)	1·7	4·0 (3·8–4·2)	*caesp. adsc.*	2 *e* ♃
VIII	23	557	17	24·9 (12–40)	38·0 (13–58)	1·5	3·8 (2·5–5·5)	*caesp. adsc.-erecta*	2 *i* III–♃
	24	558	10	27·0 (25–29)	37·2 (25–52)	1·4	8·4 (7·0–10·0)	*caesp.-adsc.-erecta*	(1) 2 *e* ♃
	25	560	12	20·2 (6–32)	37·9 (20–50)	1·9	4·0 (2·0–5·0)	*caesp. adsc.*	2 *e* II
	32	599	10	12·5 (9–18)	26·6 (19–40)	2·1	2·3 (1·5–3·0)	*pulv. adsc.*	2 *e* (*i*) II
	33	598	10	21·1 (16–27)	41·9 (28–55)	2·0	3·7 (3·0–5·0)	*pulv. adsc.*	2 *l* ♃
	34	597	15	16·8 (10–27)	38·9 (25–54)	2·6	3·0 (2·0–4·0)	*caesp. adsc.*	2 *e* III–♃
	35	607	10	35·6 (30–46)	44·0 (30–55)	1·2	7·1 (6·5–8·0)	*caesp. erecta*	(1) 2 *e* ♃
	36	611	10	18·6 (10–29)	28·1 (14–37)	1·5	3·3 (2·5–4·0)	*caesp. adsc.-erecta*	2 *e* II–♃
	49	596	10	14·0 (6–22)	34·2 (23–52)	2·4	2·9 (1·0–5·0)	*pulv. adsc.*	2 *i* ♃
	50	604	15	13·4 (8–18)	32·2 (26–39)	2·4	2·0 (1·0–3·0)	*pulv. adsc.*	2 *i–l* II–IV
	53	603	10	13·7 (8–26)	30·5 (15–49)	2·2	1·5 (1·0–3·0)	*pulv. adsc.*	2 *i–l* II–III
	60	609	10	13·1 (8–23)	35·6 (18–49)	2·7	2·2 (1·5–3·5)	*repens-pulv. adsc.*	2 *i–l* ♃
	62	610	10	15·8 (10–20)	28·0 (26–31)	1·8	2·4 (1·0–3·0)	*pulv. adsc.*	2 *e* (*i*) IV–♃
	77	575	10	34·5 (18–42)	48·0 (30–60)	1·4	5·1 (4·0–6·0)	*pulv. adsc.-erecta*	2 *i* ♃
	83	401	9	16·0 (12–19)	55·7 (45–68)	3·5	3·6 (3·0–4·0)	*caesp.-pulv. adsc.*	(2) 3 *i* ♃
	84	600	8	Not measured		Not measured		*caesp.-pulv.* (*adsc.*)	(2) 3 *e* ♃
	88	531	10	24·0 (17–29)	35·6 (22–42)	1·5	4·2 (3·5–6·0)	*caesp. adsc.*	(1) 2 *e* ♃
	130	376	12	22·0 (14–34)	58·7 (49–66)	2·7	3·0 (2·0–3·5)	*pulv. adsc.*	2 *i* (II) ♃
IX	2	885	20	21·2 (13–30)	36·5 (29–45)	1·7	4·7 (4·0–6·0)	*caesp. adsc.-erecta*	2 *e* ♃
	3	886	15	10·3 (8–13)	33·3 (25–46)	3·2	3·2 (2·0–4·0)	*pulv. adsc.* (*erecta*)	2 *e* ♃
	4*a*	887	10	23·0 (20–28)	40·1 (33–48)	1·7	3·9 (3·0–5·0)	*caesp. adsc.* (*erecta*)	2 *e* ♃
	4*b*	888	17	14·7 (10–17)	41·6 (33–48)	2·8	3·5 (3·0–4·0)	*pulv. adsc.*	2 *e* ♃
	5	889	10	23·4 (16–30)	41·8 (33–48)	1·8	4·2 (3·0–5·0)	*caesp. adsc.*	2 *e* ♃
	9	890	10	26·6 (15–37)	36·2 (28–43)	1·4	4·6 (3·5–6·0)	*caesp. adsc.-erecta*	2 *e* ♃
	71	899	12	40·4 (30–52)	70·1 (57–80)	1·7	5·3 (4·0–6·5)	*pulv. adsc.-erecta*	2 *l* ♃

grouped together with the first-year. Taking all the experiments together, the types *caespitosa* and *pulvinata* are almost equally frequent, whereas the strongly creeping *repens* type is represented by only 12% of the samples. *Adscendens* is the predominating character of the species, whereas *erecta* is less common and *procumbens* extremely rare. The ability to flower in the first season was found in 19% of the samples. Thus second-year flowering dominates, whilst flowering for the first time in the third season is exceptional. Regarding earliness, the fewest are late, and as regards length of life the perennials are clearly in the majority, there being only 8% annuals. The frequency of the many combinations has not been calculated numerically, but there is positive correlation between second-year flowering, lateness and the perennial habit. On the whole, second-year flowering plants are more often perennial than first-year (Table 4).

A calculation of the mean of the average height and breadth of the samples and of the spike length shows similar differences from one experiment to the other. For example, in Exp. III the height averages 26·5 cm. (range 15·8–41·2), whereas in V it is 22·4 cm. (range 8·3–36·4). Comparing the mean heights of all the experiments we get a variation from about 7 to 43 cm. The extreme types are rare. Most samples have a height of between 13 and 22 cm. The same thing applies to the *B*/*H* values, which range from 0·7 to 4·2, but the great majority lie between 1·5 and 2·5. The variation of the mean spike length is very considerable, from 1·1 to 8·4 cm., most of the samples having values of from 3·5 to 4·8 cm.

Table 3.

Exp. no.	Begun year		Habit-type						Development-type								
			caesp. (*caesp.*-*pulv.*)	*pulv.*	*repens* (*pulv.*-*repens*)	*proc.*	*adsc.*	*erecta* (*adsc.*-*erecta*)	1 (1–2)	2	3	*e*	*i* (*e*–*i*, *i*–*l*)	*l*	I (I–II)	II–IV (I–IV, II–(♃))	♃ II III IV }–♃
I	1938	No.	4	7	2	0	11	2	3	10	0	4	7	2	1	4	8
		%	31	54	15	0	85	15	23	77	0	31	54	15	8	31	61
II	1939	No.	1	2	0	0	3	0	0	3	0	1	1	1	0	3	0
		%	33	67	0	0	100	0	0	100	0	33	33	33	0	100	0
III–IV	1940	No.	15	2	4	0	16	5	5	16	0	3	11	7	2	12	7
		%	71	10	19	0	76	24	24	76	0	14	53	33	10	57	33
V	1941	No.	9	21	2	0	30	2	14	18	0	9	12	10	7	3	21
		%	28	66	6	0	94	6	44	56	0	29	39	32	23	10	68
VI	1942	No.	17	12	6	2	26	7	2	33	0	10	20	5	1	7	27
		%	49	34	17	6	74	20	6	94	0	29	57	14	3	20	77
VII	1943	No.	6	4	2	0	10	2	3	9	0	4	7	1	0	6	6
		%	50	33	17	0	83	17	25	75	0	33	58	8	0	50	50
VIII	1944	No.	9	8	1	0	12	6	0	16	2	9	8	1	0	4	14
		%	50	44	6	0	67	33	0	89	11	50	44	6	0	22	78
IX	1947	No.	4	3	0	0	4	3	0	7	0	6	0	1	0	0	7
		%	57	43	0	0	57	43	0	100	0	86	0	14	0	0	100
I–IX		No.	65	59	17	2	112	27	27	112	2	46	66	28	11	39	90
		%	46	42	12	1	79	19	19	79	1	33	47	20	8	28	64

4. DEVELOPMENT OF PLANTS IN NATURE AND IN CULTURE. INFLUENCE OF AGE ON HABIT AND SIZE

Linkola's studies of the development of *Prunella vulgaris* in a natural meadow locality in Finland showed that germination proceeded partly in spring and partly in autumn. The plants flowered for the first time in the fourth season, sometimes late in the third. Three whole seasons went towards building up sufficient strength for flowering to begin. In culture, 96% of Linkola's *Prunella* flowered in the second year. In explanation of the slow development in the natural habitat the author suggests root competition and the consequent under-nutrition with certain substances in the soil. Unfortunately, we do not know the behaviour in nature of forms flowering in the first year in culture. Judging from all the signs, such forms would not need such a long maturing period as the meadow race examined by Linkola.

In the experimental garden, seeds germinate in spring and later all through summer and autumn. A very large number of seedlings succeed in forming so many leaves as to be small rosette plants when winter comes. If first-year flowering Prunellas in nature germinate in a locality where root competition is small, they will undoubtedly be able to flower in the first season there too; but as a general rule, competition will cause them to flower only in the following season or the next after that. Both first- and second-year flowering plants in nature will generally winter for the first time in a maturing phase. Accordingly, the main difference between them lies in the rate of development and in the reaction to winter conditions. First-year flowering plants may flower without experiencing winter, whereas second-year flowering plants require the effects of winter dormancy in order to bloom. As a matter of fact, it has been shown that for second-year flowering *Prunella* the coming of the long days in spring is a prerequisite of floral initiation (Böcher, 1945*a*).

Molisch (1929) maintained that plants with a short 'Jugendzeit' (the period from germination to fruit setting) usually have a short life. This is verified by my studies, although here again among the types there are clear deviations from it. No. 66, uppermost in Pl. 13, and no. 81 are perennial and first-year flowering. The relation between rate of development and duration of life appears from Table 4.

Table 4. *Relation between rate of development and duration of life*

	Duration of life		
	I–II	II–IV	♃ (II) (III) (IV) }–♃
1 (1–2). First-year flowering (or both first- and second-year flowering) (%)	41	26	9
2. Second-year flowering (%)	0	30	70
3. Third-year flowering (%)	0	0	100

The brief life of the rapidly developing plants is generally caused by an excessive amount of their reserves being consumed in fruit and seed setting, so that there is little left for forming new wintering organs. In nature, where the plants have fewer flowers owing to competition, it is probable that their life is relatively longer, even after the flowering age is reached. When *Prunella* is grown in pots, where a lack of nutrition readily occurs or the further spreading of the roots is stopped, the size of the plant and the number and size of the flowers will be less (Table 6); but there will be an increase in the duration of life. I have often examined *P. vulgaris* in the autumn in its natural habitat and have only rarely seen totally withered individuals, whereas they are common in the culture experiments. In nature, most plants in autumn have the appearance shown in Pl. 14, with distinct wintering rosettes. Plants dying off on a large scale, as in the case of annuals, I have seen only in dry localities after an extremely dry summer (1947). Small new basal shoots could be seen in autumn on many culture plants which died later in the winter. These shoots, however, never succeeded in taking root sufficiently, which was undoubtedly the reason why the plants died.

In culture the plants usually flower most richly on the first occasion. This flowering signifies a culmination, for afterwards there is a decline. It is not very probable that this culmination is reached by plants in their natural habitat, because there they generally economize much more in production of shoots. There is no doubt that the decline so often observed under conditions of cultivation is due to the exhaustion of reserve materials at the first flowering, whereafter the plant rarely has time to build up another surplus for succeeding flowerings. Samples which in the first year divided into first- and second-year flowering individuals were always heterogeneous in their measured values, for the reason that some of the individuals had passed through a longer maturing time than others. Nevertheless, the morphological characters were homogeneous as a whole. There was no question of a mixing of two races, but of a segregation in a biological character within the same race. No. 98 from Germany is a good example. In 1941 (the first season), there was great heterogeneity because 40% flowered and 60% persisted as rosettes; in 1942, great heterogeneity because of the weakness of the plants which flowered in 1941; in 1943, less heterogeneity, because all the individuals were then weakened, though not to the same degree. In some instances there is also a difference in the debility in the second season, even if all the plants succeeded in flowering the first season; this is because in the first season flowering was not equally vigorous in all the individuals. For no. 97 there were two kinds of plant in the second season: weakened (height 13·2 cm. (11–16), breadth 22·7 cm. (18–27), spike length 4·5 cm. (3–5)), and unweakened (height 30·2 cm. (27–34), breadth 43·0 cm. (37–50), spike length 5·5 cm. (5–6)). A rhythm was observable in several cases. Plants which after the first flowering were debilitated and therefore in the following season became low and with few and short spikes, would sometimes in that same season reinvigorate themselves by means of basal leaf shoots, so that in the third-year flowering was stronger again. Pl. 15 illustrates a good example of weakening after flowering (no. 100) and shows the marked biological differences between the various cultures. Table 5 provides a survey of the morphological development of the plants in a number of selected samples. On the left of the table are the first-year flowering, on the right the second-year. It will be seen

Table 5. *Percentage increase or decrease in height, breadth and spike length in the second flowering year compared with the first flowering year*

First-year flowering					Second-year flowering				
No.	Height	Breadth	*B/H*	Length of spike	No.	Height	Breadth	*B/H*	Length of spike
87	−28	−11	+21	+27	121	−27	+14	+53	−21
132	−23	+4	+36	+2	119	−20	−6	+15	−5
47	−18	−34	−21	−44	73	−40	−19	+25	+9
105	−27	+3	+44	−15	80	−34	−19	+24	−47
125	−31	−37	−6	−30	78	−39	−22	+28	−21
125	−11	−9	+6	−18	103	−37	−2	+54	−19
81	+12	−2	−10	−28	74	+48	+38	−6	+4
					75	+30	+26	0	+4
55 *	+11	+32	+15	+8	42	+11	+19	+13	−2
98	−24	−30	−9	−25	124	+37	+35	0	+19
118	−26	+4	+42	−17	37	+146	+89	−22	+180
					122	+179	+120	−27	+110
Mean	−16·5	−8·0	+11·8	−14·0	Mean	+21·2	+22·8	+13·1	+17·6

* Nos. 55, 98 and 118 have both first- and second-year flowering individuals.

that there is a difference, though it is not consistent. The first-year flowering are much more disposed to weaken and decline. Marked increase is found only in some second-year flowering plants, and marked decline only in some first-year flowering plants which mostly are bi-triennial. The latter are not included in the table, because in the second year the debility was so great that I refrained from measuring the plants.

The remarkable increase of over 100 % for nos. 37 and 122 is almost unique in the material as a whole. The samples were similar in many ways, though one came from the Alps and the other from a spring fen in Jutland. I shall revert later to this similarity. Among the samples which increase in size with age are some reaching considerable values; no. 75 in the third year of cultivation attained to breadths of about 1 m., which is a record.

5. VARIED ENVIRONMENT EXPERIMENTS

(*a*) *Samples cultivated in the same year in different gardens.* In Exps. III and IV, shown in Table 2, nos. 8, 12 and 45 were cultivated in two different gardens, B and L. B lies sheltered from the wind on rather moist black mould; L is more exposed and has clayey, brownish arable soil. There was no difference in habit or development, but for no. 8 (alpine, from the Faroes) there was a distinct difference in the measurements. This sample reached the highest values on the black, wetter soil.

(*b*) *Samples cultivated in different years in the same garden.* See no. 130 in Exps. V and VIII and no. 88 in Exps. VII and VIII. In the former case there were slight differences in the measured values and none in habit or development type; but in no. 88 there is a difference in development. In Exp. VII all the plants flowered in the second year, whereas in Exp. VIII 40 % were first-year flowering. Sample no. 124 was cultivated in both Exps. VI and VII. In the former a batch of plants had spent the first summer in garden B, whence in the late summer, when in the rosette stage, they were moved to garden F. At the close of the second season these plants (124 *b*) were distinctly weaker than those which had been in the same garden all the time (124 *a*). On the other hand, there was scarcely any difference between 124 *a* (Exp. VI) and 124 *c* (Exp. VII), both of which were treated in the same manner, the cultivation year alone being different.

(*c*) *Samples cultivated in different years in different gardens.* See no. 6 in Exps. I and V, and no. 125 in Exps. III and VI. There are only slight differences in the measured values and none with regard to habit or development.

Table 6. *Difference between plants in field culture and pot culture*

No.	Development-type	H_1, Height (cm.) Exp. garden	H_2, Height (cm.) Cultivated in pots	S_1, Spike length (cm.) Exp. garden	S_2, Spike length (cm.) Cultivated in pots	Decrease in %	
						H_1–H_2	S_1–S_2
58	2 *l* ♃	22·1 (16–32)	11·2 (7–18)	4·0 (3·0–5·0)	2·1 (1·1–3·0)	49	48
57	2 *l* ♃	36·0 (19–47)	13·3 (10–17)	4·1 (4·0–4·5)	1·9 (1·2–2·5)	63	54
18	2 *l* ♃	21·1 (12–30)	4·0 (2·5–6)	5·3 (4·0–7·0)	1·0 (0·8–1·2)	81	81
48	2 *l* ♃	24·6 (16–29)	11·0 (4–18)	4·1 (3·5–5·0)	1·7 (1·3–2·2)	55	59
56	2 *l* ♃	26·5 (19–37)	13·2 (7–21)	5·1 (4·5–6·0)	2·0 (1·0–3·2)	50	61
120	2 *i* ♃	8·3 (4–12)	5·3 (3·6–7)	2·8 (2·0–3·2)	1·3 (1·0–1·5)	36	54
118	1–2 *l* (I) ♃	21·5 (18–25)	7·6 (5–11)	3·8 (3·5–4·5)	1·6 (0·8–2·4)	65	58
97	1 *l* (I) ♃	30·2 (27–34)	10·0 (7–16)	5·5 (5·0–6·0)	1·1 (0·5–1·6)	67	80
105	1 (2) *e*–*i* I (III)	19·0 (16–22)	9·9 (6–15)	3·5 (3·0–4·0)	1·9 (1·2–2·6)	48	46
119 (1)	2 *l* ♃	19·6 (12–28)	9·1 (6–12)	3·8 (3·5–4·5)	1·4 (0·8–2·0)	54	63
119 (2)	2 *l* ♃	15·7 (14–18)	5·7 (4–8)	3·6 (3·0–4·2)	1·5 (1·0–2·0)	64	58
80 (1)	2 *i* ♃	18·1 (10–25)	10·5 (6–15)	4·7 (3·5–6·0)	1·7 (1·3–2·5)	42	64
80 (2)	2 *i* ♃	12·0 (7–16)	4·2 (3–5)	2·5 (0·8–4·0)	1·4 (0·8–1·8)	65	44

(*d*) *Samples cultivated in the same year, some in the garden, others in pots* (*Table* 6). The garden plants were compared with ten potted individuals started at the same time as the garden plants. Here we find very great differences, due to the considerable difference in conditions. In all the samples examined, the heights and spike lengths of the potted plants were much smaller than those of the garden plants. The average reduction was 58 % in the height and 61 % in the spike length. Nos. 119 and 80 were examined two years in succession (1) and (2). In both cases the reduction of height from one year to the next was greater among the potted plants.

The potted plants resemble wild *Prunella* much more than do the garden culture plants. In particular, the number of flowering shoots and the length of the spike conform to those of plants in natural localities. Potting involves conditions recalling those in dense communities (root competition, shortage of nutrition).

(*e*) *Samples cultivated the same year in pots, some in light, warmth and drought, some in shade, cold and dampness.* Batches of ten pots were placed in glass frames, others in lath frames. The differences resulting from the very different conditions in the two sets of frames were very considerable (see Table 7). The differences in size and in the number of flowering shoots often exceeded 50 %. The low shoot number for the shade plants corresponds to shade modifications in *Plantago lanceolata* (Böcher, 1943). The shade plants were also much more apt to die after flowering than the light plants, which agrees with my experience of *Veronica officinalis* (Böcher, 1944); on the other hand, a good many of the light plants also died after flowering. Thus both low production of matter (shade plants) and very high production of matter lead to early death. The light plants 'over-flower', whereas shade plants do not accumulate a surplus sufficient to enable them to survive a winter after flowering.

Table 7. *Cultures in pots under different external conditions*

No.	(1) Cultures in pots, unshaded			(2) Cultures in pots, shaded			Diff. (%)		
	H_1, Height (cm.)	S_1, Spike length (cm.)	F_1, No. of flower shoots	H_2, Height (cm.)	S_2, Spike length (cm.)	F_2, No. of flower shoots	H_1–H_2	S_1–S_2	F_1–F_2
10	23 (19–27)	3·6 (3·5–4·0)	26 (21–31)	9 (5–11)	1·7 (0·5–2·4)	13 (4–21)	61	53	51
11	20 (6–28)	3·9 (2·5–4·0)	34 (24–53)	10 (6–14)	2·0 (1·6–2·6)	21 (10–36)	49	49	38
17	21 (16–26)	3·7 (3·2–4·2)	39 (25–47)	9 (7–11)	1·9 (1·1–2·6)	17 (10–22)	58	49	58
16	23 (20–25)	3·3 (2·8–3·8)	44 (41–47)	13 (9–19)	2·0 (1·4–2·6)	25 (23–26)	45	40	44
129	32 (25–44)	5·7 (3·5–7·5)	25 (12–36)	22 (11–30)	3·9 (3·0–7·0)	12 (8–20)	32	32	53
8 *a*	21 (17–24)	4·3 (4·0–4·8)	31 (20–44)	10 (6–13)	1·8 (1·3–2·0)	12 (3–21)	51	58	61
8 *b*	18 (17–20)	4·0 (3·8–4·1)	22 (17–35)	16 (14–20)	2·6 (2·4–3·0)	21 (18–23)	13	35	8
45 *a*	35 (31–38)	4·0 (3·9–4·2)	36 (29–40)	16 (11–24)	1·3 (0·6–2·3)	12 (4–20)	54	68	67
45 *b*	33 (30–37)	3·8 (3·6–4·2)	40 (33–50)	26 (23–27)	2·1 (1·5–2·3)	29 (21–35)	21	45	28
69 *a*	28 (23–32)	3·9 (3·3–4·5)	28 (23–31)	17 (13–23)	2·1 (1·9–2·4)	15 (13–16)	39	46	48
69 *b*	32 (28–34)	3·8 (3·3–5·0)	19 (14–24)	19 (17–23)	2·2 (1·6–2·6)	11 (3–26)	41	42	42
127 *a**	23 (16–30)	3·8 (3·6–3·9)	18 (13–28)	12 (8–16)	0·9 (0·3–1·8)	7 (4–10)	49	76	60
127 *b*	33 (30–35)	5·3 (4·5–5·6)	19 (13–23)	21 (17–24)	3·2 (2·5–4·0)	14 (11–17)	36	40	23

* This race normally is annual, but perennial when brought into the hothouse in winter (127 *b*). By starting with double the number of plants (20) it was possible to obtain sufficient material also of plants surviving the winter in the open (127 *a*). Here there were eight unshaded and six shaded plants capable of being measured.

A similar experiment with no. 118 from the Alps resulted in only minor differences as to height and spike length. In this case, however, the light plants were small-leaved, early and profusely flowering, the shade plants relatively large-leaved, later and with few flowers. The shade plants strengthened themselves with new rosette shoots, whereas there was only a hint of new invigoration in the light plants. In no. 18, too, the differences in leaf size between the light and shade plants were very conspicuous. Expressed in leaf breadth × leaf length (cm.) the largest leaves in ordinary pot cultures (*p*), in unshaded (*u*) and shaded (*s*) frames gave the following values: 3·2 (*p*), 3·0 (*u*) and 3·8 (*s*), whereas the majority of the leaves gave values of 1·4 (*p*), 0·7 (*u*) and 2·5 (*s*).

(*f*) *Samples treated as in* (*e*), *but also exposed to different temperatures in the winter season.* This experiment was made with nos. 8, 45, 69 and 127, and was an expansion of the foregoing (Table 7, lower part). There were forty plants (sixty of no. 127) of each sample. Half of them (*b*) spent the whole winter in a greenhouse at about 5–8° C., the others (*a*) remained in the open. When spring came the greenhouse plants were put back into frames, shaded or unshaded. With the exception of no. 69, the difference between unshaded and shaded plants was greater for the plants which had been in the open the whole time. This, of course, was due to the fact that by being brought indoors in winter the shade plants were compensated in a way for the reduced production of matter in summer. For the unshaded plants of nos. 8, 45 and 69 there was scarcely any difference in the height and spike-length measurements between the two batches with different winter temperatures. On the other hand, there is a pronounced difference in this respect as regards the southern European no. 127. This plant is generally an annual, and the few survivals of the open-air batches were greatly weakened at the beginning of the second season. The number of flowering shoots gives an impression of the flower amplitude. Here we find reduced flower ring in the unshaded greenhouse batches of nos. 8 and 69. The mild winter in the house induced the plants to put out new leaves under short-day conditions, which presumably had a reducing effect on the setting of flowering shoots. No. 56 was particularly pronounced in this respect, for, as mentioned separately in Böcher (1945*a*, Table 2), it failed to set spiked shoots at all if it spent the winter indoors; the same applied to no. 58.

Taking it all round, *Prunella vulgaris* is a plant that is easy to modify. In particular, its height, spike length, number of flowering shoots, and other quantitative characters are much influenced by fluctuations of external conditions, whereas the physiological-biological characters and habit-type are not affected so much. It requires an operation as radical as the removal of winter cold to make the annual no. 127 become a perennial. The cultures in the unshaded frames were exposed to much more drought and heat than those in the ordinary pot cultures, and this sometimes precipitated the termination of the life of the plants or, in some instances, caused perennials to become biennials because they exhausted themselves and did not succeed in forming new wintering rosettes.

6. VARIATION IN RELATION TO GEOGRAPHIC DISTRIBUTION

The material in Tables 1 and 2 can be applied to an examination of the variation in relation to the geographical distribution of *P. vulgaris*. It must not be forgotten, however, that to obtain material enough we must take all the individual experiments together. This will inevitably involve certain errors if we concentrate on the measurements, whereas the uncertainty will be small if we take the characters embodied in habit- and development-types. This is clear from the foregoing varied environment experiments. I shall therefore keep mainly to habit- and development-types.

The geographical origin of the material is shown in Table 1. Grouping some of the regions together we obtain seven main areas, A–G:

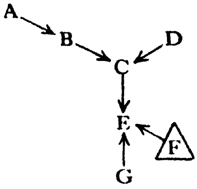
(A) North-west and western Europe: 15 cultures, nos. 1–14.
(B) Jutland: 30 cultures, nos. 15–44.
(C) Danish islands: 32 cultures, nos. 45–76.
(D) Sweden, Baltic States, Russia: 13 cultures, nos. 77–91, but excluding nos. 85 and 79.
(E) Central and south-east Europe: 23 cultures, nos. 92–114.
(F) Alpine countries: 10 cultures, nos. 115–124.
(G) Mediterranean countries: 11 cultures, nos. 125–136.

The relative occurrences of thirteen different groups of characters ranging under habit- and development-types are put together in Table 8. In a number of diagrams

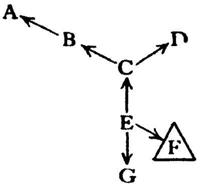
(p. 303) I have illustrated characters which reveal interesting differences in the various areas. There are steady increases and decreases in certain directions, especially south to north, and north to south.

Table 8. *Percentage distribution of habit- and development-types in seven geographical areas (A–G)*

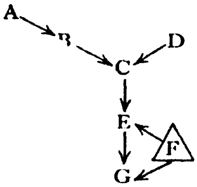
Area	*caesp.* (*caesp.-pulv.*)	*pulv.*	*repens* (*pulv.-repens*)	*pro-cumbens, adscendens*	*erecta* (*adsc.-erecta*)	1 1–2	2 (2)–3	*e*	*i* (*e–i, i–l*)	*l*	I I–II	II–IV	♃ II, III, IV –♃
A	73	27	0	87	13	0	100	67	27	7	0	27	73
B	60	30	10	77	23	10	90	43	33	23	3	40	57
C	20	60	20	77	23	22	78	13	61	26	10	26	65
D	46	46	8	92	8	23	77	39	46	15	0	23	77
E	13	57	30	100	0	26	74	13	57	30	17	17	65
F	80	20	0	90	10	20	80	30	50	20	0	20	80
G	73	27	0	64	36	45	55	30	60	10	30	50	20



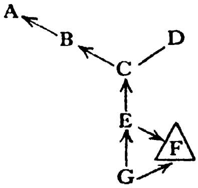
repens
pulvinata
'late'



caespitosa
'early'



1 and 1–2
I–II



2 and (2)–3
♃

In these diagrams F (the alpine countries) is drawn in a triangle for the purpose of showing that many of the samples are from highland localities. As the chief result it can be shown that *repens*, *pulvinata* and 'late' go together with an accumulation of the particular genes in central Europe, and that conversely, *caespitosa* and 'early' are coupled, with the highest frequency in northern, montane and southern areas. Further, there is a clear increase in first-year flowering and annual habit in a southern direction. Expressed in the terminology of Huxley-Gregor this means that in *P. vulgaris* we have demonstrated a number of topoclines corresponding to the four diagrams. It may perhaps cause some surprise that *caespitosa* and 'early' are more prominent both in northern-montane and in southern areas, but for earliness this is easy to understand. The northern and montane samples behave like northern or alpine races of other species (cf. Turesson, 1925, 1930; Clausen, Keck & Hiesey, 1940); they must flower and set fruit in the relatively short vegetation period allotted to them. The southern plants must also be early, however, because they have to flower and seed before the summer drought really becomes perceptible. Otherwise there is no agreement between the northern-montane and the southern. The former are typical, second-year flowering and perennial, the latter to a great extent are first-year flowering and not perennial. The point must not be lost sight of, however, that this rule applies only to those plants when cultivated in Copenhagen. I am unable to say anything definite as to the duration of life of these races in the south. There is no doubt that there, too, many plants will die away in the summer drought; on the other hand, the mild winter will not weaken the plants much, and therefore Prunellas which are annuals in Denmark will probably in many cases be perennials in the south (cf. p. 302).

There seems to be an especially high frequency of the *repens* character in central Europe, and another of *erecta* in Denmark and southern Europe. This, however, must be interpreted with caution, for in actual fact a specially large number of samples was collected in meadow localities in central Europe and a good many from woodlands in Denmark, which may easily have an influence on the result. However, there was no deliberate selection of localities when collecting the samples either in Denmark or in central Europe, for which reason there is a possibility that in the various regions the species occurs with different frequencies in the plant communities available to it. Thus, when all is said there are undoubtedly ecological differences behind the aforesaid topoclines, a circumstance on which we shall throw more light in the following section.

At four places in Europe collections were made of samples at different heights above sea-level: the Faroe Islands, Norway, the Alps and Portugal. In the Faroes, both lowland and highland samples belonged to the same habit-type, and they were all perennials; but among the highland samples there was a greater tendency for some plants to die after too luxuriant flowering. The principal difference lay in the assimilation period of the spike-bearing shoots and in earliness. The assimilation period for the flowering shoots is long for lowland plants and short for montane subalpine samples. In midsummer these plants on the whole have withered or already abscissed leaves on the shoots. In the matter of earliness, the highland plants are 8–10 days earlier than the lowland plants (Pl. 16, fig. 1). There is no clear difference in the height of the plants (Table 2, Exps. III and IV, nos. 7 and 8).

From Norway, Exp. IV has three samples, one of them from a subalpine locality. It was a little earlier than the other two and shed the leaves of its shoots much earlier than the lowland plants.

From the Alps there are samples in both Exp. V and Exp. VI. Those in Exp. V are especially interesting, because they give us an impression of the clines which can be observed from the lowland up into the mountains. It is true that no. 121 is a fairly high-growing type (see Pl. 13, top, cult. no. 308), but it was distinctly subalpine in its earliness and perennial habit and in early loss of leaves on the flowering shoots. No. 120 was very low; a small, dense, often rather flat tussock with very short spike-bearing shoots. No. 122 was also very low. Low growth in conjunction with the *caespitosa* character is distinctly a montane-subalpine combination, but this low growth is not a general feature of the subalpine samples. For nos. 118 (600 m. above sea-level), 119 (700 m. above sea-level), 120 (1100 m. above sea-level) and 121 (1930 m. above sea-level), the aforesaid clines from lowland to highland may be expressed thus:

	118		119		120		121
Habit	*pulv.*	→	*caesp.-pulv.*	→	*caesp.*	→	*caesp.*
Development	1–2	→	2	→	2	→	2
Earliness	*l*	→	*l*	→	*i*	→	*e*
Height (cm.)	29	→	20	→	8		(22)

From Portugal there are a lowland plant which in culture is an annual (no. 127), and a montane plant from 950 m. above sea-level (no. 128, mentioned p. 292). Compared with no. 127 and nearly all other samples, no. 128 developed with marked slowness; it did not succeed in flowering the first season, and it died during the first winter.

The most general subalpine character combination is undoubtedly low growth, *caespitosa-adscendens*, second-year flowering, early, and perennial. This combination characterizes the subalpine ecotype of the species. Nevertheless, it bears such a remarkable resemblance to a number of northern races from Iceland, the Faroes, Norway and Värmland (no. 82) that it is justifiable to speak of a boreal-subalpine ecotype. This boreal-subalpine character combination is not entirely confined to the north and mountains, for we find related combinations in several other places. No. 253 from Budapest is like no. 8 from the Faroes, though it is much larger and an annual. In Denmark we have similar types in three instances: no. 20 from the western slope of Bulbjerg on the Skagerrak, no. 24 from a wind-eroded western slope on the Limfjord, and no. 37 from a spring-fen in Randbøldal in central Jutland. The latter was quite boreal-subalpine in appearance, with low stature and early withering of the spiked shoots. It is scarcely fortuitous that this combination occurs in a spring-fen, where many species with a northern distribution can grow. The Bulbjerg plants are also rather low but not perennials, and no. 24 differs by its extremely long spikes. In this respect it beat all records (Pl. 16, fig. 2). And perhaps it is no accident either that exposed western slopes in north-west Denmark accommodate types with almost boreal-subalpine character combinations.

The lowland Prunellas embrace an enormous number of races. The combinations among them that are capable of being separated out into ecotypes will be referred to in the next section. Among the southern European samples there are some which, as regards earliness and habit, recall boreal-subalpine plants as already stated (see, for example, nos. 134 and 135); but there are also extremely tall *erecta* races (e.g. no. 133 from Trieste) and the very large-flowered, long-stemmed no. 129 from Madrid. This race is also different in respect of its leaf type; it has long-stalked, narrow almost lanceolate leaves. The leaf form, the height, the spike length and other characters are very reminiscent of no. 137 from North America; like the latter, no. 129 must be placed to Fernald's var. *lanceolata*. This variety represents a group of races with distinct association with America. According to the collections in the Botanical Museum in Copenhagen, however, it is also found in East Asia, Australia, southern Europe and Morocco. Hultén (1930) refers to the range of var. *lanceolata* as 'American transgressing into eastern Asia'. It is evident that as regards the shape of its leaf *P. vulgaris* forms a topocline with an accumulation of 'lanceolate' genes in America.

Among the samples from the coasts of the Baltic no. 79 from Öland and nos. 83, 84 and 142 from Gotland are in a separate category. In Exp. VIII, nos. 83 and 84 on the whole did not bloom until the third season; the same is true of the one plant of no. 142 that was cultivated in a pot. Thus their development was extremely slow. They persisted as flat, dense cushions and had short floral shoots. No. 79 flowered in the second season, but in habit it resembled the others. It grew slowly and could be characterized as *caespitosa* with short spike shoots (almost *erecta*). The height was 4·7 cm. (4–6 cm.), the breadth 8·7 (7–10 cm.) and the spikes 0·9 cm. (0·8–1·0 cm.). As regards earliness it was intermediate and its length of life was three to many years. Some of the individuals of no. 83 had particularly large flowers, clear evidence of hybridization with *P. grandiflora*, which is frequent in those very areas, Öland and Gotland. It looks very much as if we could separate a low, slowly developing alvar-ecotype* of *P. vulgaris*; to do so definitely, however, will require more material and also an investigation of the possible significance of an admixture of *grandiflora* genes; for it is fairly certain that *P. grandiflora* differs from *P. vulgaris* not solely in the character of the flowers, but also in habit-type and development-type (see p. 308).

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* Alvar = steppe-like grassland areas on thin soil overlaying limestone rocks on the island Öland in the Baltic.

7. Variation in relation to habitat

The material in Table 2 has also been applied to an examination of the variation in relation to the ecological conditions at the locality of the plants. Here again Exps. I–IX are put together, particular weight being attached to habit- and development-types. Naturally, there are many different kinds of habitat, but we cannot here go into the finer details and must confine our examination to the following groups:

(*a*) Dry grasslands and pastures, dry roadsides, etc., in lowland areas (Denmark, central Europe). Nos. 20, 24–26, 28, 39, 47, 63–66, 100, 105.

(*b*) Less dry grasslands and pastures, roadsides, etc., in lowlands areas (Denmark, central Europe). Nos. 17, 21, 23, 31–34, 38, 45, 50, 54–56, 61, 67–68, 77, 97, 101, 109, 111.

(*c*) Wet meadows and ditches in lowland areas (Denmark, central Europe). Nos. 15, 16, 18, 19, 29, 30, 37, 46, 48, 49, 57, 58, 60, 75, 93–96, 102, 103, 106.

(*d*) Woodlands and shady places in lowland areas (Denmark, central Europe). Nos. 35, 36, 40–43, 59, 62, 64, 69–74.

(*e*) Subalpine or montane meadows in the Faroes, in Norway and the Alps. Nos. 6, 8, 10, 119–124.

The result of this examination is shown in Table 9. As was the case with the geographical distribution, there are very great and clear differences. If preliminarily we take each character separately, we get a number of clines which here, in conformity with Gregor, we may call ecoclines. The series *a*→*b*→*c* from dry to wet localities shows a decrease for *caespitosa* and an increase for *repens*, a decrease for first-year flowering and a duration of life to 1–2 years, as well as for early flowering and an increase for lateness. The series light-open localities (*a*–*c*)→woodlands and shady localities (*d*) shows a strong increase for *caespitosa* and *erecta*. Table 9 also includes the samples from montane-subalpine meadows (*e*). The materials from lowland meadows and highland meadows differ, especially as regards earliness and vegetative propagation.

Table 9. *Percentage distribution of habit- and development-types, mean height, etc., in five habitat groups* (*a*–*e*)

abi- tat	*caesp.* (*caesp.-pulv.*)	*pulv.*	*repens* (*repens-pulv.*)	*pro-cumbens-adsc.*	*erecta* (*adsc.-erecta*)	1 (1–2)	2	*e*	*i* (*e*–*i*) (*i*–*l*)	*l*	I I–II	II, III, IV	♃ II III IV –♃	Mean height (cm.)	*B*/*H* mean	Mean spike length (cm.)
(*a*)	42	50	8	92	8	67	33	58	33	8	50	17	33	20·6 (14·7–27·0)	2·2 (1·4–2·8)	4·4 (3·3–8·4
b)	23	64	13	87	13	9	91	18	50	32	0	32	68	21·3 (11·7–35·2)	2·2 (1·4–3·9)	3·4 (1·7–5·1
c)	10	57	33	100	0	5	95	5	48	48	0	14	86	18·6 (6·6–36·2)	2·6 (1·9–4·2)	3·0 (1·1–5·3
d)	60	33	7	27	73	13	87	40	47	13	0	33	67	26·4 (13·4–41·3)	1·6 (0·8–2·6)	4·0 (2·4–7·1
e)	100	0	0	89	11	0	100	67	22	11	0	11	89	17·1 (8·3–24·3)	1·8 (1·3–2·7)	3·4 (2·1–4·8

The table also comprises averages of the means (and range of the means) for height, *B*/*H* value and spike length. As these measured values are highly modifiable, and as the averages apply to all the Exps. I–IX put together, the figures must be treated with a certain caution. Nevertheless, there are several differences so marked that they must be regarded as significant. This applies, for instance, to the marked height and the low *B*/*H* value for the group from woodlands (*d*) and the considerable spike length in the group from dry localities (*a*).

In accordance with the above, a separation can be made between the following character combinations, each of which marks its habitat. In the lowlands we have at least four ecotypes:

Dry-soil ecotype: caespitosa-pulvinata, adscendens, 1 (1–2), *e*, I–II, rather long-spiked.
Semi-wet soil ecotype: pulvinata, adscendens, 2, *i*, II–♃.
Wet soil ecotype: pulvinata-repens, adscendens, 2, *i–l*, ♃, short-piked, broad, low.
Shade ecotype: caespitosa, erecta, 2, *e–i*, II–♃, fairly long-spiked, tall, narrow.

Each ecotype is characterized by the most frequent combination of characters of the type of habitat in question. It is quite another matter that in localities that are closely related ecologically there are often widely different combinations, and that the above denotative character combinations are not nearly always present in the various samples, for very often they differ from the norm in respect to one or more of the characters. Accordingly, these ecotypes are theoretical to some extent; on the other hand, it is obvious that they must be theoretical when they have to apply to a highly fluctuating variation.

Whereas it is easy enough to comprehend the biological significance of rapid development and short life of plants living on dry soil, it is less easy to understand the significance of the distribution of several of the other characters. Nevertheless, the straight, upright growth of group (*d*) in shaded localities seems comprehensible enough, for the leaves become well separated and thus shade each other less. However, a wholly prostrate, strongly creeping plant may also form a leaf mosaic, and we find one in fact among the samples of group (*d*). No. 70 is quite low and creeping, and in this resembles the wet-soil ecotype, but it is early and first-year flowering, just the characters that do not conform to the most frequent combination on wet soil. I consider it very probable that this sample, as well as one or two others like it, belongs to a separate, creeping, woodlands ecotype corresponding to Timbal-Lagrave & Jeanbernat's var. *silvestris* (see Böcher, 1940, pp. 7–9). This variety is creeping and is considered to be associated with woodlands. The erect, robust woodlands ecotype (Pl. 13) does not seem to have received any official designation, and yet it was collected by the Danish botanist Svend Andersen on 19 July 1925 in a thicket on the island of Taasinge, and on the label called f. *nemoralis* mihi. Andersen's plant is 60 cm. high and erect, and yet so narrow-leaved that it recalls var. *lanceolata*. I consider it pretty certain that in culture this plant would also prove to be erect and tall. Further, in the herbarium of the Copenhagen Botanical Museum there are specimens of such *erecta* plants from the woodlands Jonstrup Vang and Gribskov in Denmark, from a wet alder forest in Latvia and from the Kazan district in Russia. As I have said, it is probable that we must also reckon with a low, creeping, woodland ecotype. If, accordingly, we take the two lowest and most creeping samples out of group (*d*), the average height of that group will rise to 28·5 and the average B/H value will fall to 1·4.

It is obvious that on studying more material there is a chance of finding other combinations within the various habitat groups to justify the setting up of still more ecotypes.

8. RELATION BETWEEN *PRUNELLA VULGARIS*, *P. LACINIATA* AND *P. GRANDIFLORA*

Two of the cultivated samples, no. 117 from Graz and no. 123 from Arnoldstein, Austria, usually have whitish yellow flowers and deeply incised leaves and are more hairy than the others; therefore they belong to var. *laciniata*, or, if it is desired to consider the type as an independent species, *P. laciniata*. In any case, *P. laciniata* represents a geographically separate character combination, associated with south-eastern Europe and Asia. There is evidently no sharp boundary between *P. vulgaris* and *laciniata*, and spontaneous hybrids between the two are described by many authors. Among my cultures there was one, no. 228 (not included in Tables 1 and 2), which was particularly interesting in this connexion. The sample was sent me as seed from a botanic garden under the name of var. *laciniata*. Ten seedlings were planted out, and in the first year none of them got beyond a rosette. Eight of them had normal rosette leaves, two laciniate rosette leaves. In the following year all ten flowered, and there appeared a curious segregation into seven violet and three white flowered, and, as regards the leaves of the shoots, three with deeply laciniate, six with less laciniate leaves and one with simple leaves. This segregation was strongly indicative of a hybrid between *P. vulgaris* and *laciniata* and of this hybrid being fertile.

P. grandiflora is not sharply separated from *P. vulgaris* either, though in this case the differentiating characters are more obvious. Spontaneous hybrids occur wherever the two species meet, and as a matter of fact *P. grandiflora* and *laciniata* also cross readily in nature. I have not undertaken any crossing experiments, having left this for a later occasion. But I have had the opportunity to cultivate several samples of *P. grandiflora*, and, as in the case of *P. laciniata*, found segregations which bore evidence of hybridization. The four samples cultivated, nos. 144–147, are shown last in Table 1. Two, nos. 145 and 146, were cultivated in Exp. V, and two, nos. 144 and 145, in Exp. VIII. One sample, no. 147, was also tried in Exp. VI, but only four plants germinated from the seeds, and therefore I preferred to keep the plants in pots. All samples of *P. grandiflora* in culture proved to be second-year flowering, perennial, and distinctly *caespitosa-adscendens*. Sylvén (1906) also mentioned second-year flowering plants from Sweden. The samples I tested bore a great morphological resemblance to subalpine-montane forms of *P. vulgaris*, especially the three from the Alps.

Nos. 144–146, cultivated in a garden, were subjected to a close examination. In all three the material segregated into typical *grandiflora* individuals and individuals very like *P. vulgaris*. At Kyndby Strand in Zealand, the only place in Denmark where *P. grandiflora* now grows naturally, there is a possibility of crossing with *P. vulgaris*, and, indeed, the herbarium contains the hybrid. In the Alps, too, there is ample opportunity for hybridization. No. 146 was sent me as '*P. grandiflora*, perhaps the hybrid *P. grandiflora* × *vulgaris*'.

Table 10. *Cultivation of three samples of* Prunella grandiflora, *showing a segregation of* vulgaris-*like individuals* (*marked b*)

Year	Sample no.	No.	Height (cm.) mean and range	Breadth (cm.) mean and range	*B/H* mean and range	Length of spike (cm.) mean and range	Length of flower (cm.) mean and range
1942	145*a*	6	24 (19–38)	40 (32–54)	1·7 (1·4–2·1)	4·4 (3–5)	2·0 (1·7–2·2)
	145*b*	4	18 (17–20)	41 (36–43)	2·2 (2·1–2·4)	3·7 (3–5)	1·7 (1·6–1·8)
	146*a*	13	16 (11–22)	23 (19–27)	1·4 (1·2–1·8)	4·5 (3–6·5)	2·1 (1·9–2·3)
	146*b*	3	21 (18–25)	48 (44–54)	2·3 (2·2–2·4)	4·7 (4–5)	1·8 (1·7–1·9)
1945	145*a*	10	24 (18–27)	42 (25–62)	1·8 (1·4–2·3)	5·5 (4–6·5)	2·3 (2·2–2·4)
	144*a*	19	18 (9–25)	32 (20–47)	1·8 (1·4–2·8)	4·0 (3–6)	2·1 (1·8–2·4)
	144*b*	1	14	37	2·6	3·0	1·6 (1·5–1·6)

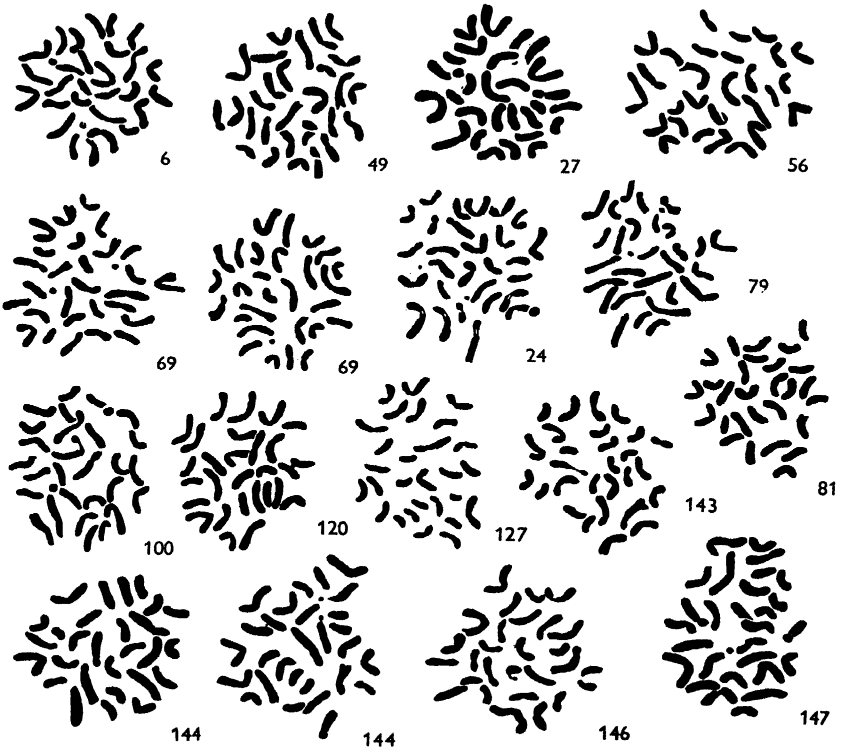
The material in Table 10 indicates that in some cases there is positive correlation between small flowers and large plant breadths. For no. 146 this was quite evident; the small-flowered individuals resembling *P. vulgaris* were *pulvinata*, all the others being *caespitosa*. In itself the length of the flower is not the most characteristic feature, for there are specimens, though rare, of *P. vulgaris* (no. 129), reaching right up to the flower measurement of *P. grandiflora* without resembling it otherwise at all. It is more the relation between calyx and corolla and the curve of the corolla tube that distinguish the two species. However, these characters vary too, and one may find every kind of transition from the one to the other in regard to the calyx-corolla relation and the nature of the corolla tube. In general it may be said, however, that *P. grandiflora*'s largest flowers are 2–2·3 cm. long, whereas the largest of *P. vulgaris* are 1·0–1·6 cm. long. The mean of the average flower lengths of twenty-five samples was 1·3 for *P. vulgaris*. The two samples of *vulgaris* with the largest flowers were both Mediterranean, viz. no. 129, average length 2·0 (1·6–2·2) and no. 125 with 1·6 (1·3–1·8).

With so few cultures it is impossible to compare the variation ranges of *P. grandiflora* and *P. vulgaris*. If the herbarium can tell us anything about this, it looks as if *P. vulgaris* varies more. *P. grandiflora*'s variation is not small, but part of the variation is undoubtedly due to hybridization with the other two *Prunella* species. There is a distinct difference between the Danish lowland type, which has fairly deeply incised almost pinnately lobed leaves, and the three from the Alps which have simple ovate leaves, and here again there is a considerable difference between the two samples from 700 m. altitude (nos. 145 and 146) and no. 147, which came from a point 200 m. above the timberline and in all 1900 m. above sea-level and there grew in an Alpine 'mat' together with *Primula minima* and *P. auricula, Draba aizoides* and *Gentiana acaulis*. It was extremely low, actually a 'Polsterpflanze', forming a dense flattened hemisphere and having short spikes on stems a few centimetres long rising just a little above the leaf cushion. This undoubtedly is an alpine ecotype of the species, whereas nos. 145 and 146 belong to a montane and no. 144 to a lowland ecotype. By their very close leaf cushions and low stature the large-flowered individuals of no. 83 from Gotland (referred to above, p. 305), which were certainly of a hybrid nature, were mostly reminiscent of the alpine type.

9. CYTOLOGICAL INVESTIGATIONS

Hruby (1932) found the chromosome number $2n = 32$, for *Prunella vulgaris, laciniata* and *grandiflora*. He fixed in Navashin's fluid and stained with Heidenhain's haematoxylin. The illustrations seem to have been drawn from very clear metaphase plates in root tips; he also has a figure of meiosis in *P. laciniata* with 16 bivalents. In my preliminary paper (Böcher, 1940) I reported having verified Hruby's count by examining root-tip mitoses of four samples of *P. vulgaris* from widely different localities. My preparations, however, were not wholly good, especially because the gentian-violet staining had not been quite successful. One thing I realized quite early, that Hruby's remark 'it is of interest that there are no differences of shape and size of chromosomes in these three species' would not apply in all cases, for my material contained both long and short chromosomes. In the hope of finding chromosome-morphological differences between the many forms of *Prunella* I started—ably assisted by Kjeld Holmen—on a somewhat more extensive investigation. This led to the rather surprising result that *P. vulgaris* from twenty-seven different stations had the number $2n = 28$, and that *P. grandiflora* from three different stations also had $2n = 28$. Improved technique had led to this discovery. Only the most vigorous roots from newly repotted plants were used. The root tips were fixed in Navashin's fluid, and staining was by means of Newton's iodine-gentian violet method, but with gentian violet from the Anilin Co. of New York. A very long staining time of 24 hr. was employed, as this period alone permitted of a sufficiently lengthy differentiation in alcohol-clove oil. Only vigorous root tips contained mitoses in which the chromosomes lay sufficiently free of one another, and only satisfactory staining made it possible to study finer details such as the position of the centromeres and the trabants. The cause of the earlier mistakes was that some large bent-over chromosomes had each been counted as two. Hruby's drawings showed no bent ones, and therefore the lighter staining observable in some cases at the centromere was assumed to be the space between two chromosomes. Hruby's figures show rather wide separations between the chromosomes, but he has not put in a single primary constriction or trabant, which suggests that his technique was not of the very best. Possibly he may also have mistaken some primary constrictions for spaces between chromosomes. There remains the problem of Hruby's meiosis figure with its 16 bivalents. I have not myself examined *P. laciniata*, but I am inclined to think that there has been some mistake here too. Hruby's mitosis figure of this species differs in no way from the others, and in the meiosis figure itself the bivalents seem to have been looked at rather obliquely from above, which with long and perhaps already separated bivalents may perhaps lead to an incorrect idea of the number.

My search for chromosome-morphological differences gave if anything a negative result. In most of the samples of both species there were about four specially short chromosomes. In the mitosis plates of nos. 6, 100, 120 and 81 (Text-fig. 1) the difference in size seems to be less pronounced than in the other samples. In some samples there is also a greater number of V-formed chromosomes than in others. As a rule there are two sat.-chromosomes of medium length, but trabants are observable only when the technique has been particularly successful, and the connexion to the chromosomes to which the satellites are attached is mostly invisible. Thus the possibility is not precluded that there may be chromosome-morphological differences, but probability argues that they are small if they do occur. There seems to be far-reaching cytological stability and homogeneity in *P. vulgaris* and its nearest relatives.



Text-fig. 1. Mitoses from root tips of various races of *Prunella vulgaris* (upper three rows) and of *P. grandiflora* (fourth row). The numbers refer to the consecutive numbers in Table 1. No. 143 is *P. vulgaris* var. *lanceolata*, from Montreal. In all cases the somatic chromosome number is 28. × 2870.

One circumstance was fairly characteristic of the material as a whole. The nucleolus was retained unusually long during mitosis, sometimes as far as the metaphase. It was nothing uncommon to see mitosis metaphases in side view, where the nucleolus hung like a sack from the equatorial plane in the direction of one of the poles.

In *P. vulgaris*, $2n = 28$ was found in nos. 6, 7, 18, 24, 27, 42, 49, 51, 56, 69, 75, 77, 78, 81, 83, 84, 88, 100, 120, 126, 127, 129, 138, 139, 140, 141, 142, 143.

In *P. grandiflora*, $2n = 28$ was found in nos. 144, 146 and 147.

10. DISCUSSION AND CONCLUSIONS

Together with *P. grandiflora* and *laciniata*, *P. vulgaris* forms a coenospecies with $2n = 28$. Although they seem quite capable of exchanging genes and of forming fertile hybrids, the three species must be regarded as good species, i.e. ecospecies, each consisting of a number of subordinate units. In the present case these are called ecotypes. The principal ecotypes in *P. vulgaris* are a montane-boreal ecotype, and at least four lowland ecotypes associated with dry, medium dry, wet and shaded soil respectively. If we simply accept the subordinate units as ecotypes, there seems to be close agreement with the terminology and the opinion as to the variation of the species expressed by Turesson and later by Clausen *et al.*

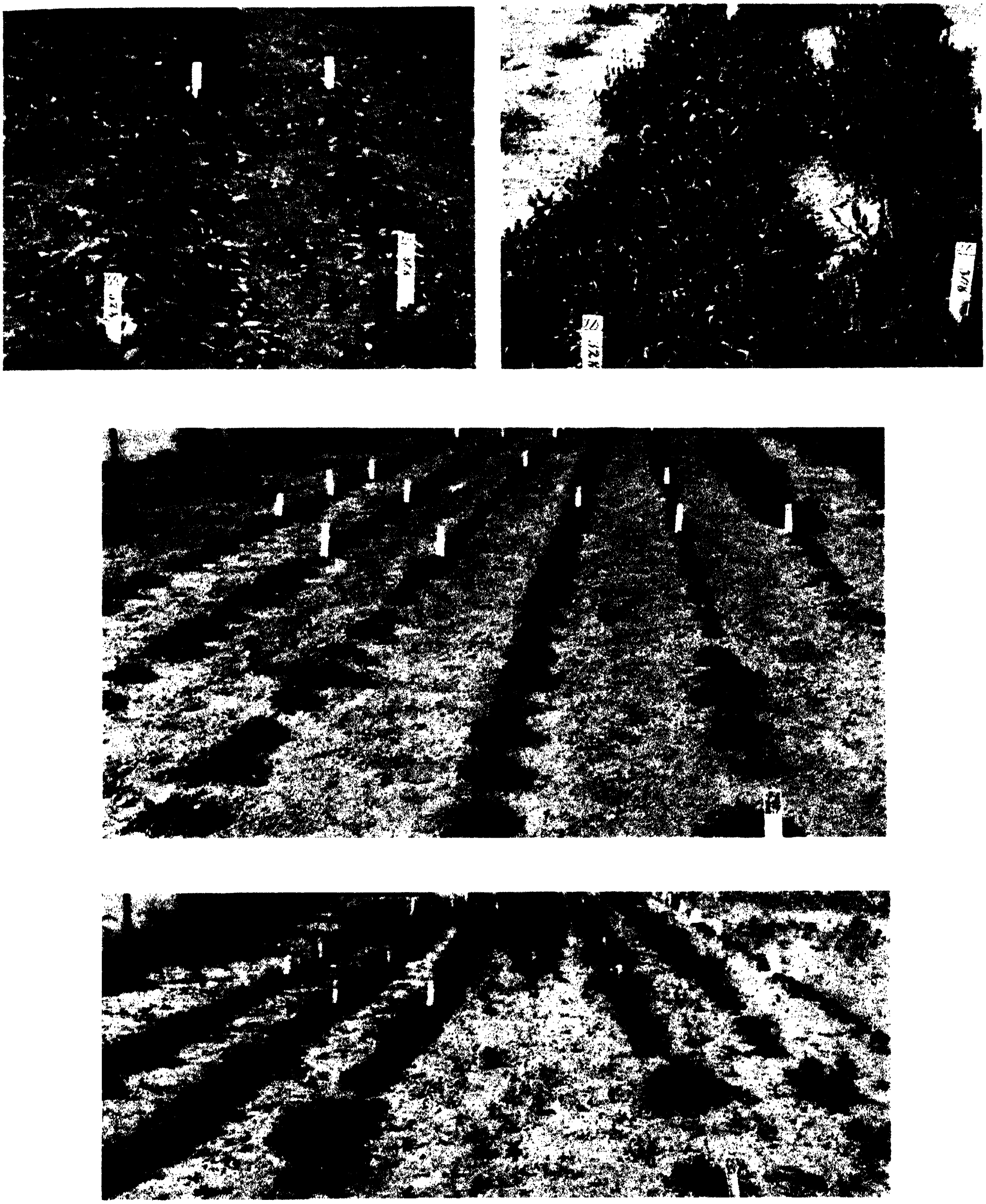
It would seem, however, that in *P. vulgaris* there is a continuous variation, for which reason the ecotypes in question represent the most frequent character combinations in the different types of habitat. A large number of biotypes or races may not be conformable to the ecotype system, because in respect of one or more characters they do not agree with the ideal combination (the most characteristic features of the ecotype). These biotypes or races may, of course, be regarded as intermediate forms between the ecotypes; but as there seems to be continuous variation, they are not hybrids between well-defined units. On the present basis it is not impossible that the character combinations which are styled ecotypes may be harmoniously stabilized gene-complexes, and that crossing would make them relatively disharmonic gene-combinations. If so, these harmonic complexes would figure as subordinate systematic units almost as an approach to what Huxley understands as 'dependent subspecies'. In that case the variation would no longer be wholly continuous, even if between these initial dependent subspecies there are broad zones of intergradation with a lively exchange of genes. However, the extraordinarily large number of different character combinations appearing from Table 2 seems to argue that the variation is entirely continuous.

It must be borne in mind, however, that the ecotypes of *Prunella*, representing the most frequent character combinations in a given environment, are really theoretical concepts. They are composed of ranges within a whole series of continuous or almost continuous character gradients or clines. And the boundaries of these ranges are not demarcated by any zones of rapid change. I have made a selection of some geographical areas (A–G, Table 8) and some groups of habitats (*a–e*, Table 9), and within them examined the relative occurrence of the characters. The numerical material of the tables shows sudden leaps from one area to another and from habitat to habitat, but this is merely because in selecting these groups of areas and habitats an artificial stepping down within the clines is inevitable. If one had material enough and could make the geographical areas small and subdivide the habitat groups into finer plant-sociological units, the steps would be lower and perhaps smoothed out. Let us take *caespitosa* as an example of a character. Its value drops from north-west Europe (73) to Jutland (60) and on to the Danish islands (20). Calculating north and south Jutland separately, we get 73—64 (north Jutland)—58 (south Jutland)—20, or a stepping down with more but lower steps.

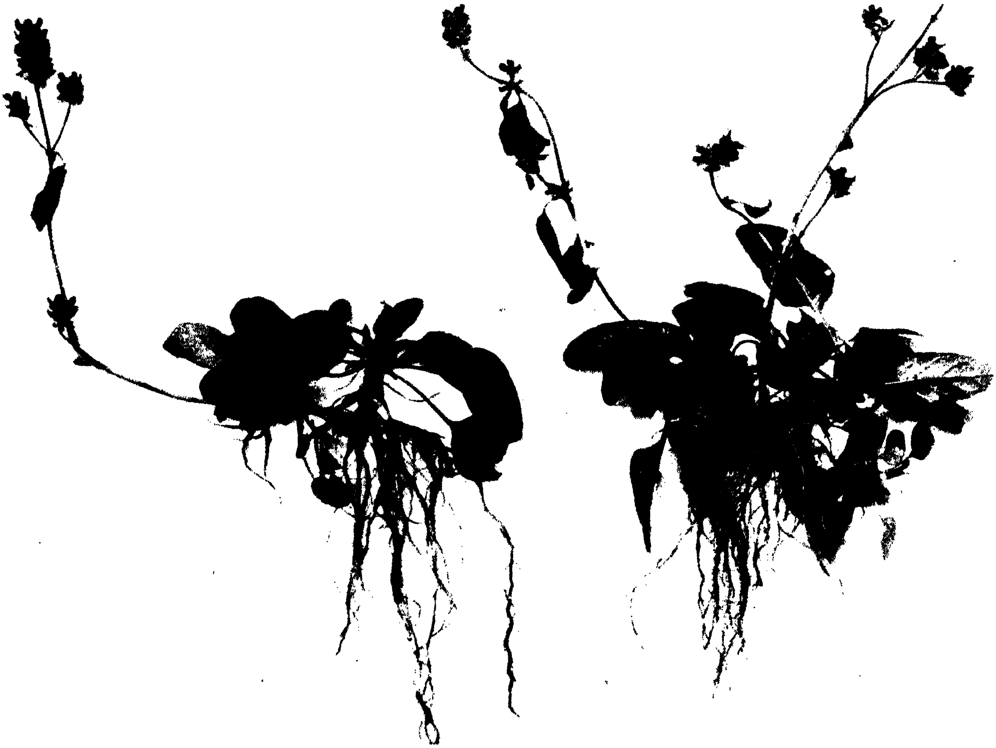
It is open to discussion whether we should operate with ecotypes in the case of continuous variation (cf. Huxley, 1945, p. 226), whether we should not preferably confine ourselves to a description of the existing clines. Viewed from the angle of evolution genetics it is of course clear that ecotypes, which are arbitrarily delimited, ideal combinations of characters, are of no significance whatever, being merely ranges of clines. But from the viewpoint of practical taxonomy, ecology and plant geography, this kind of ecotype is of a certain importance. It is an advantage to be able to single out the most representative character combination in a given locality and give it a name. But the reader must not be left in doubt that the name does not correspond to a well-defined unit. Huxley suggests setting up the cline as an independent systematic unit co-ordinated with a term like subspecies. This, however, must be of lesser importance, as clines concern the species as a whole. In order to express the essentials with regard to the variation of a species he suggests e.g. the addition of 'Size S.–N.; melanin E.–W. from desert belt to sea, then S.W.–N.E.'. An addition of this sort, however, would only be useful when the whole species was being described. In describing a local flora, for instance that of Iceland, it is of significance for *P. vulgaris* to be able to add: especially in the boreal-montane ecotype (*caesp.*, *adsc.*, 2, *e*, ♃), or, in the case of dry grassland, to add: especially in the form of the dry-soil ecotype, which is first-year flowering and annual/biennial.

Both Turesson and Clausen *et al.* (1940) operate with much more well-defined ecotypes which behave almost as subspecies and indeed often receive that appellation, and which hybridize with one another where they meet. Apparently there is a distinct difference between these ecotypes and the *Prunella* ecotypes, although a closer examination of the matter indicates that it is more a matter of degree. As to Turesson's investigations, they are often based upon rather few cultivations from widely different habitats. Such experiments will always show extremes, and it is impossible to judge to what extent the extreme types are connected with a continuous series of intermediate races. Actually, it is probable that many of the plants which Turesson has cultivated will, on a closer study, prove to behave like *Plantago maritima* or *Prunella vulgaris*. As regards the species examined by Clausen and his co-workers in California, they grow in a country where from the coast towards the interior there are close belts with climates of very different characters. The climate changes from one extreme to another within a distance of a few miles. Here it is evident that the ecotypes may frequently be sharply divided one from the other, and any clines may be broken up into fairly distinct subspecies on account of geographical barriers. The climate also changes in the north European lowland, but not suddenly; here the variation is more likely to be continuous. Therefore the difference existing between well-defined ecotypes and the undefined *Prunella* ecotype must be due to the presence or absence of sharp climatic boundaries.

The ecotypes found by Clausen *et al.* are not homogeneous either. Within the alpine ecotype of *Potentilla gracilis* some races or biotypes are frost-resistant, others very early, others again both. The ideal character complex in this case is the combination of frost resistance and earliness, but the alpine ecotype is also permitted to include those biotypes which possess only one of the characters. The three authors in fact write: 'the individual ecotype presents a complex picture. It may, however, be statistically distinguishable from other ecotypes in characters of importance for survival in a given habitat'. My *Prunella* ecotypes are separated by just such biologically important characters. Thus, all in all there is no profound difference between the ecotype concept which has crystallized



BÖCHER—RACIAL DIVERGENCES IN *PRUNELLA VULGARIS* IN RELATION TO HABITAT AND CLIMATE



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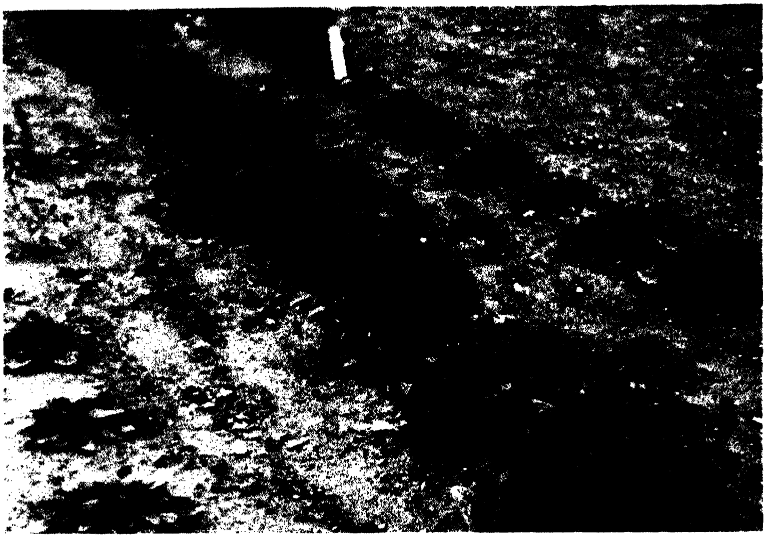

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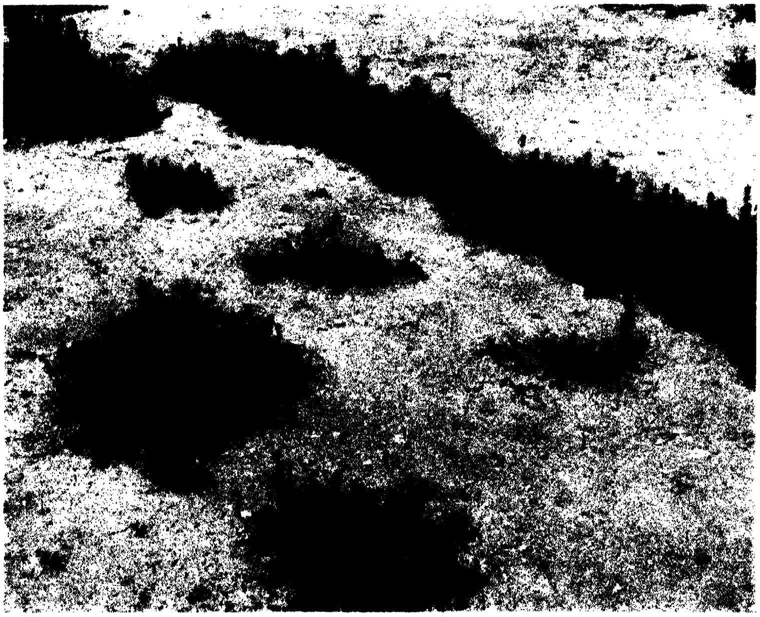

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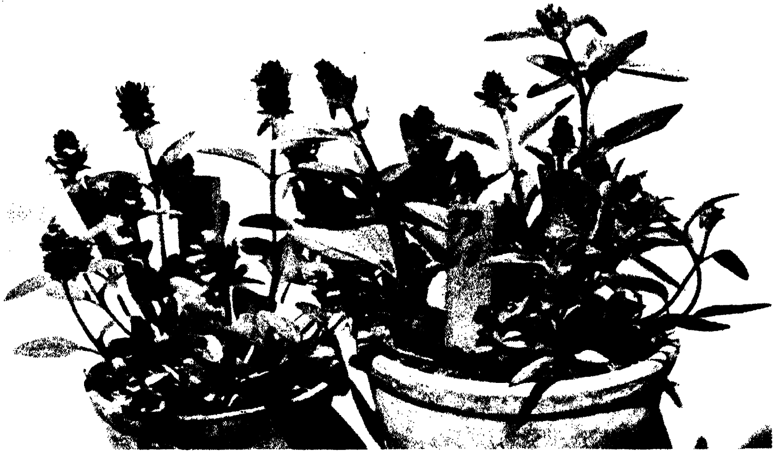

Fig. 1

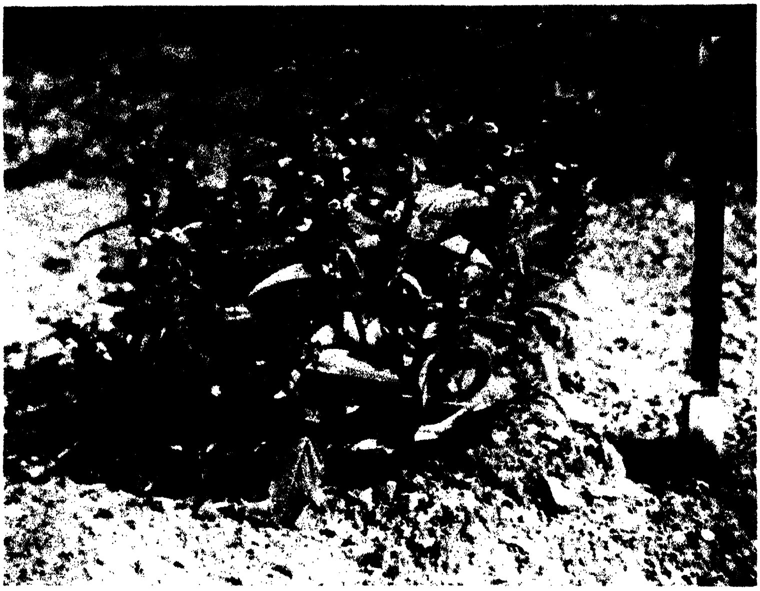

Fig. 2

BÖCHER—RACIAL DIVERGENCES IN *PRUNELLA VULGARIS* IN RELATION TO HABITAT AND CLIMATE

through the investigations in California and the investigations made by Gregor and the present writer. In the case of the *Achillea millefolium* complex which was described recently by Clausen *et al.* (1948) the vast amount of variability and the rather continuous variation remind a good deal of the behaviour of *Prunella vulgaris*.

REFERENCES

BÖCHER, T. W. (1940). Introductory studies on variation and life-forms in *Prunella vulgaris*. *Dansk. bot. Ark.* **10**, no. 3.

BÖCHER, T. W. (1943). Studies on variation and biology in *Plantago lanceolata* L. *Dansk. bot. Ark.* **11**, no. 3.

BÖCHER, T. W. (1944). The leaf size of *Veronica officinalis* in relation to genetic and environmental factors. *Dansk. bot. Ark.* **11**, no. 7.

BÖCHER, T. W. (1945*a*). Some experiments to elucidate the influence of winter conditions on shoot development and floral initiation on various races of *Prunella vulgaris* and *Ranunculus acer*. *Dansk. bot. Ark.* **12**, no. 3.

BÖCHER, T. W. (1945*b*). Beiträge zur Pflanzengeographie und Ökologie dänischer Vegetation. II. *Kgl. danske vidensk. Selsk. Biol. Skrifter*, **4**, no. 1 (with an English summary).

CLAUSEN, J., KECK, D. & HIESEY, W. M. (1940). Experimental studies on the nature of species. I. Effect of varied environments on western North American plants. *Publ. Carneg. Instn*, no. 520.

CLAUSEN, J., KECK, D. & HIESEY, W. M. (1948). Experimental studies in the nature of species. III. Environmental responses of climatic races of *Achillea*. *Publ. Carneg. Instn*, no. 581.

FERNALD, M. L. (1913). The indigenous varieties of *Prunella vulgaris* in North America. *Rhodora*, **15**, 179.

GREGOR, J. W. (1930). Experiments on the genetics of wild populations. I. *Plantago maritima*. *J. Genet.* **22**, 15.

GREGOR, J. W. (1939). Experimental taxonomy. IV. Population differentiation in North American and European sea plantains allied to *Plantago maritima*. *New Phytol.* **38**, 293.

HOFFMANN, H. (1887). Culturversuche. *Bot. Ztg*, 1887, no. 46, p. 754.

HRUBY, K. (1932). Cytologie a anatomie československých Brunell. *Preslia*, **11**, 40. Praha.

HULTÉN, E. (1930). Flora of Kamtchatka and the adjacent islands. IV. *K. svenska VetenskAkad. Handl.* **8**, no. 2.

HUXLEY, J. (1945). *Evolution, the Modern Synthesis*. London.

IRMISCH, TH. (1856). Beiträge zur vergleichende Morphologie der Pflanzen. V. *Abh. Naturf. Ges. Halle*, **3**, 63.

LINKOLA, K. (1935). Über die Dauer und Jahresklassenverhältnisse des Jugendstadiums bei einigen Wisenstauden. *Acta for. fenn.* **42**, no. 2.

MOLISCH, H. (1929). *Die Lebensdauer der Pflanze*. Jena.

SYLVÉN, N. (1906). Om de svenska dikotyledonernes första förstärkningsstadium eller utveckling från frö till blomning. I. *K. svenska VetenskAkad. Handl.* **40**, no. 2.

TURESSON, G. (1922). The genotypical response of the plant species to the habitat. *Hereditas, Lund*, **3**, 211.

TURESSON, G. (1925). The plant species in relation to habitat and climate. *Hereditas, Lund*, **6**, 147.

TURESSON, G. (1930). The selective effect of climate upon the plant species. *Hereditas, Lund*, **14**, 99.

EXPLANATION OF PLATES

PLATE 13

Top: Exp. V, no. 66 (culture no. 328) from Denmark, and no. 121 (culture no. 308) from the Alps, photographed in September 1941 (left) and September 1942 (right). No. 66 is *repens-pulvinata, adscendens*, 1 (2) *l* ♃, whereas no. 121 is *caespitosa, adscendens*, 2 *e* ♃. Middle and bottom: Exp. VI in August 1942 and June 1943. In the centre of the middle picture the vigorous rosettes of no. 85 from Finland; in the bottom picture all the plants of this number are dead. On the left of these, in both pictures, is no. 93; it only becomes wholly prostrate in the second year (bottom picture). There are several fairly low and creeping types from meadows on the left of the pictures, but also an *erecta* type from woodland (no. 73) farthest back in the extreme left row. Right middle, a first-year flowering *caespitosa* type no. 135 (culture no. 324) from Urbino, Italy, and in the second row to the right of the middle another first-year flowering, no. 125 from Lisbon. In the second season the latter is weaker than in the first.

PLATE 14

Prunella vulgaris. Two individuals from the wood Kongsøre Skov, Zealand, photographed 9 November. Both have a distinct wintering rosette. They have no great vegetative power of production, and compared with cultivated plants there is little spike setting. Photo. Sophus Franck.

Plate 15

Culture experiments with ***Prunella vulgaris***. Above 1941, below 1942. From left: no. 1 (culture no. 333) from Iceland (2 *e* II–IV), no. 100 (culture no. 311) from dry field in Thüringen (1 (2) *i* I–III), and on right: no. 58 (culture no. 294) from moist meadow at lake Arresø, Zealand (2 *l* ♃).

Plate 16

Fig. 1. Pot culture with no. 8 (culture no. 69) and no. 7 (culture no. 68). Culture no. 69 is subalpine and early flowering; culture no. 68, lowland, has just begun to flower. Photo. 1941.

Fig. 2. Example of alpine habit in a sample from a wind-exposed western slope in Denmark (no. 24, Næsby Dale, Limfjord). The steel measure on the right drawn out to 20 cm. The plant is extremely long-spiked. Photo. 1945.

GLUTAMINE IN THE METABOLISM OF BARLEY PLANTS

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(With 5 figures in the text)

I. Introduction

The role of the acid amides, asparagine and glutamine, has attracted the attention of plant physiologists and biochemists ever since the classical researches of Schultze and Prianischnikow. A comprehensive review and reinterpretation of much of the earlier work has been made by Chibnall (1939), with particular regard to the relation between the amides and the metabolism of proteins in plants. More recently it has been found that glutamine plays an important part in the metabolism of both animals and micro-organisms: mainly in this connexion Archibald (1945) has reviewed the chemical properties and physiological role of the amides.

In higher plants it is generally considered that the two amides are physiologically similar, the predominance of one or the other depending chiefly on the species. A survey of their distribution led Schwab (1936) to suggest that the activity of specific enzymes, asparaginase and glutaminase, was an important factor determining the relative abundance of the amides. He proposed a distinction between 'asparaginase' and 'glutaminase' plants. There is evidence, however, that the balance between the amides may be decisively affected by physiological conditions in a way which cannot be readily interpreted along the lines indicated by Schwab. For example, the two amide fractions, now known to be mainly asparagine and glutamine, show consistent differences in relation to catabolism of proteins and respiration of starved barley leaves. Glutamine accumulates most rapidly at first in the freshly detached leaves, while asparagine is only formed at later phases of starvation (Yemm, 1937). In other species distinctive features have been observed and discussed by Vickery (1939), Mothes (1940), Steward (1946) and their collaborators.

An attempt has been made here to analyse more fully the physiological conditions which control the formation of the amides in barley leaves. The evidence now available shows that glutamine, as distinct from asparagine, is readily formed under conditions of normal growth and development of the plant; the unstable amide appears to be an important intermediary in the assimilation and translocation of nitrogen and in the interconversion of amino acids. In a final section the possibility is examined that glutamine may occupy a key position in the mechanisms which co-ordinate the metabolism of proteins and carbohydrates with cell respiration.

II. Formation of glutamine and asparagine in detached leaves

It is found that the accumulation of amides in barley leaves may be decisively influenced by the following conditions:

(i) The concentration of readily available carbohydrate.
(ii) Illumination of the leaves.

(iii) Oxygen concentration.

(iv) Stage of development of the plant and age of the leaves.

An account of the available data is given in this section, together with a brief consideration of the relation between the formation of amides and the catabolism of proteins in the leaves.

(1) *The influence of carbohydrate concentrations*

For experimental purposes, a wide variation of carbohydrate concentration in leaves of similar age and cultural history may be brought about either by harvesting them at different times of the day or night, or by artificially darkening the plants for a short preliminary period. Both of these methods have been used in experiments during the growing seasons 1936, 1938, 1939. These experiments show uniformly that, when samples of leaves differing in their carbohydrate content are detached and subjected to starvation, the accumulation of glutamine during the initial phase is substantially increased in leaves containing large amounts of carbohydrate. Asparagine, by contrast, is formed much more rapidly in those leaves which contain relatively small amounts of sugar when they are detached from the plant. The data may be illustrated by reference to a typical experiment carried out in 1938. Six samples of leaves were collected at different times between 4 p.m. 2 August and 10 p.m. 3 August, from plants of the Plumage Archer variety grown under normal field-crop conditions in the Bristol Botanic Garden. Some of the plants were darkened artificially by means of a black cloth during 3 August in order to obtain a sample of leaves of very low sugar content. Each sample was subdivided at random into two halves, the first being analysed immediately to estimate the initial concentration of carbohydrates and nitrogenous constituents. The second part was kept under starvation conditions for 48 hr. before subjecting it to analysis. Controlled conditions of starvation and analytical methods were similar to those already described (Yemm, 1935, 1937). The results are summarized graphically in Fig. 1 in order to show the relation between initial concentration of carbohydrates and the accumulation of glutamine and asparagine in the detached leaves.

Total amide nitrogen was produced in greatest amount in the leaves having a low concentration of sugars, an effect attributed mainly to the accumulation of asparagine which represented 83% of the amides formed under conditions of minimum sugar content. In the leaves of higher sugar content the amount of glutamine often exceeded that of asparagine. It may be noted at this point that the breakdown of tissue protein was not appreciably affected by sugar concentration: the loss of protein-N (see Table 3) varied from 3·25–3·78 mg./g. fresh weight in the different samples, the variations falling within the limits of experimental error and showing no correlation with sugar content. The rate of respiration of the leaves was, on the other hand, very clearly influenced by sugar concentration both initially and during the course of starvation. This effect of sugar content on the metabolism of the leaves will be considered more fully on another occasion.

(2) *Effects of illumination on the formation of amides*

When detached leaves are exposed to continuous artificial illumination, as opposed to starvation conditions, glutamine accumulates rapidly in the tissues. At light intensities sufficiently high to reduce the losses of carbon dioxide by respiration to a negligible level, experiments have shown that the increase of amides is attributable almost exclusively to the formation of glutamine.

Several different methods have been used in experimental work of this kind. Usually the leaves were arranged as a single layer in a shallow glass-fronted chamber which could be sealed and immersed in a thermostatically controlled water-bath. A stream of moist air, freed from carbon dioxide, was passed through the chamber and light supplied by a battery of electric lamps. By means of appropriate analyses the formation of amides was estimated in samples of illuminated leaves and compared with similar analyses of leaves kept under starvation conditions. In other experiments the leaves were allowed to stand with their cut surfaces in water in a room at 19–20° C., a water-screen being inserted between the lamp battery and the leaves in order to reduce heating effects.

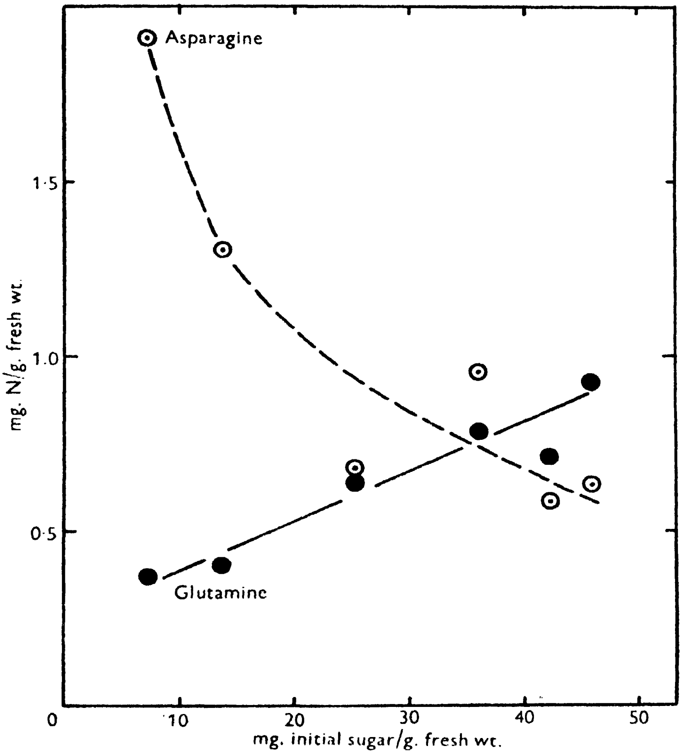


Fig. 1. The influence of carbohydrate concentration on the formation of glutamine and asparagine in starving leaves. Increases of the amides during the first 48 hr. of starvation are plotted against the initial sugar content of the six samples of leaves collected at different times of the day or night, 2–3 August 1938.

The results of an experiment, in which the former method was used, are summarized in Table 1 to show the changes of soluble nitrogenous constituents which occurred in illuminated and starved leaves after 60 hr.

As shown by the increase of total soluble nitrogen, the breakdown of protein proceeded almost as rapidly in the illuminated leaves as in the starved ones, but the nature of the amides formed differed widely. In the illuminated leaves over a ninefold increase of glutamine was observed, accompanied by only a small accumulation of asparagine. The sample of starved leaves, on the other hand, contained more asparagine than glutamine. Judged by the total amount of amides and ammonia formed, protein catabolism was more extensive in the illuminated leaves; there are indications that this was at the expense of amino-acids.

Other experiments, in which lower light intensities were used, gave results of an intermediate character, but it was always found that glutamine accumulated to a greater extent in the light than in the dark. Somewhat similar effects of light on the accumulation of amides in detached tobacco leaves have been described by Vickery & Pucher (1939). Taken in conjunction with the results set out in section II (1), it is probable that the effects of illumination operate by maintaining the carbohydrate of the leaves at a higher level, although light may have a more direct effect on the formation of organic acid precursors.

Table 1. *Protein catabolism in illuminated and starved leaves*

Nitrogen fractions	mgms. N/gm. fresh weight			Δ (2–1)	Δ (3–1)
	Initial sample (1)	After 60 hr. starvation (2)	After 60 hr. illumination (3)		
(1) Ammonia N	0·04	0·33	0·14	0·29	0·10
(2) Glutamine N	0·18	0·60	1·68	0·42	1·50
(3) Asparagine N	0·16	0·72	0·20	0·56	0·04
(4) Other amino N	1·31	2·36	1·80	1·05	0·49
(5) Total soluble N	3·61	6·25	6·00	2·64	2·39

Table 2. *The effect of anaerobic conditions on the formation of amides*

Nitrogen fractions	mg. N/g. fresh weight				
	Initial sample	Starved in nitrogen		Starved in air	
		24 hr.	48 hr.	20 hr.	44 hr.
Ammonia N	0·07	0·07	0·07	0·06	0·07
Glutamine N	0·07	0·09	0·08	0·46	0·75
Asparagine N	0·08	0·08	0·11	0·09	0·43
Other amino N	1·04	1·06	1·17	1·45	1·49
Total soluble N	2·26	2·13	2·49	3·20	4·26
Total insoluble N	5·30	5·16	4·52	4·00	3·40

(3) *Oxygen concentration and formation of amides*

The investigation of the effect of oxygen concentration on the metabolism of the detached leaves was undertaken for the chief purpose of gaining further information concerning the respiratory mechanisms. Concentrations of oxygen higher than that of air have relatively little effect on the rate of respiration, but at very low concentrations both the respiration and the catabolism of proteins are affected. Under anaerobic conditions the formation of amides is almost entirely suppressed in freshly detached and in highly starved leaves.

Data collected in an experiment in which a direct comparison was made between leaves starved in air and in pure nitrogen are summarized in Table 2.

The samples analysed after 24 and 48 hr. in nitrogen showed that the breakdown of tissue protein was practically stopped under these conditions; the accumulation of amide was scarcely appreciable even after 48 hr. starvation. An analysis of the effects of very low oxygen tensions on the metabolism of mature barley leaves is complicated by the fact that injury to the tissue rapidly occurs, and is detectable after 24 hr. under anaerobic conditions. It is probable, therefore, that small changes of nitrogenous constituents observed after 24 hr. in nitrogen are partly of an autolytic nature.

The results described in this section are in sharp contrast to those recorded by Mothes (1933) for *Phaseolus multiflorus*, but in agreement with those of Paech (1935), and more recently (Wood, Cruickshank & Kuchel, 1943; Wood, Mercer & Pedlow, 1944*a*; Wood & Cruickshank, 1944*b*) for other species.

(4) *Stage of development of the plant and age of the leaves*

A systematic study of these effects has not yet been made, but data collected during the seasons 1935–9 indicated clearly that both factors were important, especially in controlling the rate of glutamine formation in freshly detached leaves. In each season most of the experiments were made with leaves developed just preceding, or contemporary with, the emergence and development of the ear. Samples of leaves taken from the same field crop have consistently shown that the rate of accumulation of glutamine gradually increased as the season advanced. The highest rates were observed in the final 'flag' leaf of the shoots, but there was evidence that the penultimate leaf also formed glutamine more rapidly as it aged. Experimental data collected during 1935 and 1938 are summarized in Fig. 2 to show the amounts of glutamine accumulating during the initial stages

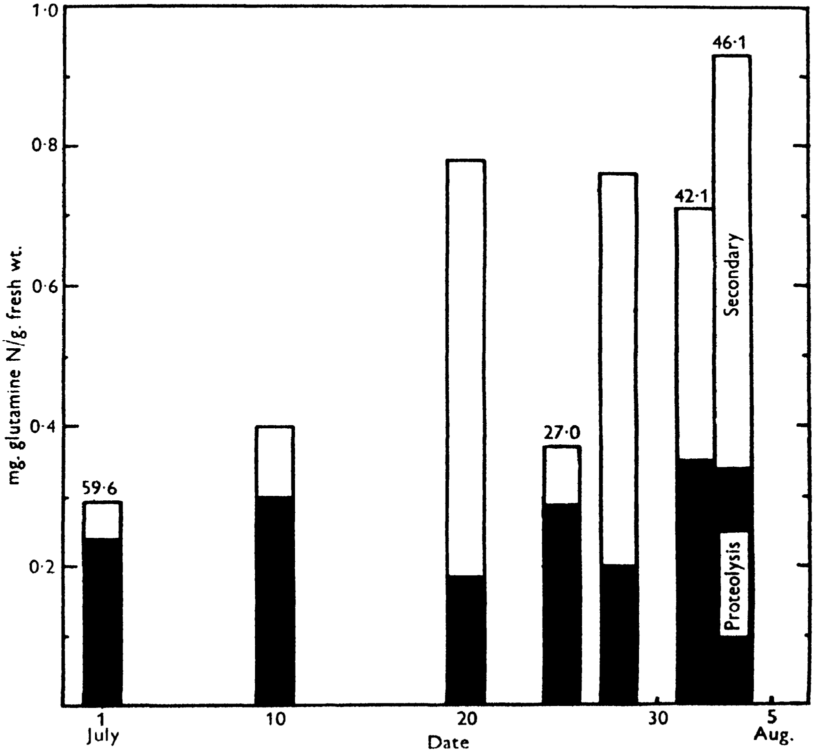


Fig. 2. The formation of glutamine in samples of leaves detached on different occasions July–August, 1935 and 1938. The height of the columns represents the amount of glutamine formed in the leaves during the first 48 hr. of starvation, and the solid part is an estimate of the maximum amount of glutamine which could be derived from proteolysis, i.e. loss of protein nitrogen × 0·094. The data of 1938 are distinguished by the figures at the heads of the columns: these figures record the mg. fermentable sugar per g. fresh weight in the leaves when they were collected.

of starvation in leaves harvested on different occasions, between 1 July and 3 August. As will be discussed more fully in the next section, it was possible to estimate the *maximum* amount of glutamine which could have arisen by proteolysis as distinct from secondary changes. Such an analysis of the results showed that the greater part of the amide in the older leaves was formed by secondary changes, as shown in the diagram.

The possibility of attributing the acceleration in the rate of glutamine formation entirely to differences in the concentration of carbohydrates is eliminated by the analyses of the samples collected in 1938. As shown in Fig. 2, the highest level of soluble sugars was found in the samples of leaves collected on 1 July, whereas glutamine accumulated much more slowly in these leaves than in later samples.

(5) *Amides and the catabolism of leaf proteins*

A breakdown of tissue protein accompanied the formation of amides in all these experiments with detached leaves, and it is important to decide whether the amides could arise simply by hydrolytic changes, or whether more extensive catabolism and interconversion of amino-acids was implied. In an earlier discussion of this problem the suggestion was made that glutamine might arise mainly by proteolysis, while asparagine was a secondary product formed under conditions of carbohydrate starvation. A more certain decision on this point is now possible; leaf proteins have been separated from mature barley leaves, and partial analysis have established their close similarity with representative preparations of protein of some grass leaves, described by Chibnall (1939). The methods of preparation and analysis are fully described elsewhere (Yemm, 1948), but in the present connexion the general result may be summarized.

In relation to the formation of glutamine and asparagine, chief interest centres in the amide nitrogen and dicarboxylic amino-acids of the leaf protein. The best estimates of these fractions are as follows:

	Total protein N (%)
Amide N	4·7
Glutamic acid N	8·0
Aspartic acid N	4·9

There is, as yet, no evidence as to whether the amide nitrogen of the protein is combined with glutamic or aspartic acid residues, but assuming that it is exclusively linked with one or other of these amino-acids, an estimate can be made of the *maximum* amount of glutamine or asparagine which could be formed simply by hydrolysis of the protein. A re-examination of already published and additional data on this basis has given a clear indication that both glutamine and asparagine may arise by secondary changes in the detached leaves.

During the rapid accumulation of glutamine, which followed detachment of older leaves rich in carbohydrates, the increase of amide nitrogen was commonly two to three times greater than could be accounted for by hydrolysis of protein. Similarly, the asparagine formed in the more highly starved tissues must have been largely of secondary origin. An analysis of the data described in section II (1) gives in Table 3 a typical example of the experimental results leading to this conclusion.

The different samples analysed in this series gave strong evidence that the amide nitrogen of both glutamine and asparagine was formed in greater amount than could be derived from the hydrolysis of the leaf proteins. In absence of a full identification of the amino-acids of the freshly detached leaves, it was often impossible to decide with certainty whether glutamic and aspartic acids, as well as the amide nitrogen, were of secondary origin. However, the very large amounts of glutamine and asparagine, which were found in illuminated and highly starved leaves respectively, leave little doubt that glutamic and aspartic acids were formed secondarily; under these different circumstances the individual amides often represented more than half the total soluble nitrogen derived from protein catabolism (see Tables 1 and 3).

Table 3. *Origin of amides in leaves of varying carbohydrate content*

Sample no.	mg./g. fresh weight				
	Initial sugar content	Loss of protein N	Amide N from proteolysis	Amide N formed	
				Glutamine	Asparagine
1	42·1	3·78	0·18	0·36	0·29
2	25·3	3·25	0·15	0·32	0·33
3	13·7	3·25	0·15	0·21	0·65
4	36·1	3·68	0·17	0·39	0·48
5	46·1	3·65	0·17	0·47	0·32
6	7·3	3·45	0·16	0·21	0·96

The relation between glutamine and protein catabolism is of particular interest. A secondary formation of this amide can often be detected within 12–24 hr. of detaching the leaves, and it is evidently increased under conditions of relatively high carbohydrate concentration and in illuminated leaves. These observations clearly suggest that glutamine may be readily formed in the normal metabolism of the growing plant, and a brief review of other evidence bearing on this point is given in the next section.

III. GLUTAMINE AND THE METABOLISM OF NITROGEN DURING GROWTH AND DEVELOPMENT

Gregory (1937), Richards (1936) and their collaborators have shown that an important characteristic of the metabolism of nitrogen in barley plants is the extensive movement of nitrogenous constituents from senescent tissues to developing and meristematic parts. The final phase of the annual cycle of development is the transfer of nitrogen to the grain, where it is stored mainly in the reserve proteins of the endosperm.

Some experiments carried out in this laboratory during the growing season 1947 have shown how the withdrawal of nitrogen from vegetative parts dominated the metabolism of protein during the late phases of growth when the grain was developing. On six occasions between 22 July and 26 August, samples of fifty complete shoots were collected at random from a small plot of Spratt Archer barley grown in the Bristol Botanic Gardens. Each sample was subdivided for analysis into the chief vegetative parts (leaves, stem and root) and the grain; the results are set out in Fig. 3 to show the changes in the distribution of nitrogen during ripening of the plants. The earliest sample was collected when the ears were just emerging from the sheaths and the tillers carried four to five green leaves. At first there was evidence of an appreciable assimilation of nitrogen and a steady increase in the nitrogen of the grain was accompanied by a loss from leaf blades, sheaths and stem. The results indicated that at least 69% of the nitrogen grain reserve was derived from the vegetative parts of the shoot. Of this 55 % was mobilized from the leaves and awns, and the rapid withdrawal of nitrogen from these organs is shown in Fig. 4. The view that the total nitrogen of the tissues is the resultant balance between synthetic and downgrade activities (Mothes, 1933; Gregory & Sen, 1937), implies that a greater proportion of the nitrogen of the grain may have been derived from vegetative parts than is indicated by these analyses.

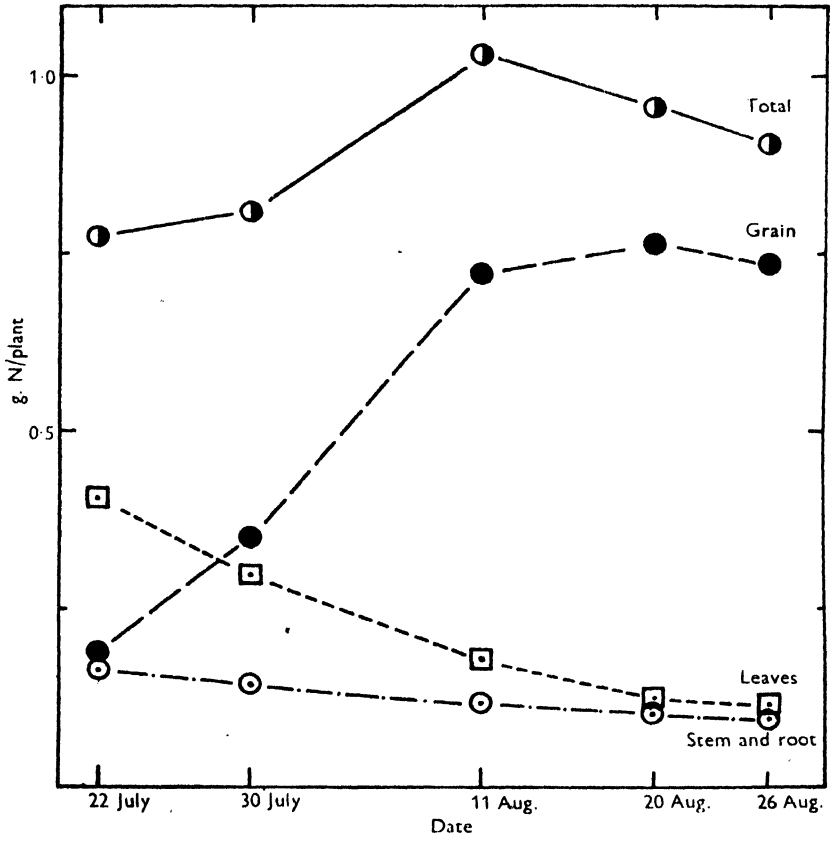


Fig. 3. Changes in the distribution of total nitrogen between the grain, leaves, stem and root during the development of barley plants, 22 July–26 August 1947.

Considered in terms of the metabolism of protein, there is little doubt that the changes in the distribution of nitrogen involve an extensive breakdown of cytoplasmic and chloroplastic proteins of the leaves and other vegetative parts and their translocation to the grain, where they are condensed mainly as the reserves of the endosperm. It is instructive, therefore, to compare the composition of the reserve proteins with that of the vegetative tissues. In common with other cereals, the proteins of the barley endosperm contain relatively large amounts of glutamic acid and amide nitrogen, which, from the work of Damodaran (1932*a*) and Damodaran, Jaaback & Chibnall (1932*b*) are known to be combined mainly as glutamine in the intact protein. It may be estimated that nearly half the total nitrogen of hordein, the prolamin of barley, is in the form of glutamine, while relatively high amide values have been reported for the reserve glutelin, hordenin, by Csonka & Jones (1929) and Lamour (1927). These structural features of the endospermic proteins are in sharp contrast with those of the leaf proteins (see section II (5)) and clearly suggest that an extensive interconversion of amino-acids occurs, leading to the formation of the large amounts of glutamine incorporated in the reserve proteins. The fact that the formation of glutamine during catabolism of proteins reaches a climax in older leaves, contemporary with the development of the ears, gives strong circumstantial evidence that we are here dealing with the normal cell mechanism whereby the interconversion is brought about.

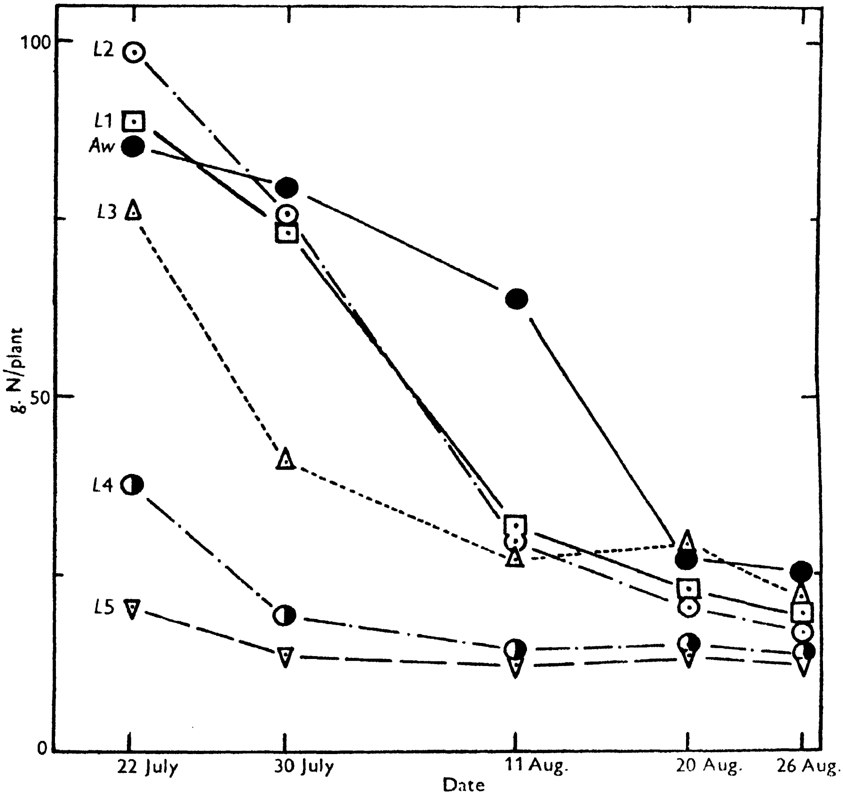


Fig. 4. Loss of nitrogen from leaves and awns during the later stages of development. Functional leaves are numbered successively *L*1–*L*5, *L*1 being the final or 'flag' leaf developed in the shoot. Awns are indicated by *Aw*.

Some observations made by Richards & Templeman (1936) with barley plants grown under varied conditions of mineral nutrition support this view. It was shown that the amounts of total amide nitrogen in the leaves closely reflect the metabolism of protein. Under conditions favouring a rapid breakdown of tissue protein, such as in senescent and potassium deficient leaves, there was an increase of amide nitrogen, while the much reduced synthesis of protein in phosphate-deficient leaves was associated with very marked accumulation of amides. Richards & Templeman do in fact suggest from their data that a conversion of soluble nitrogenous compounds to amides may be a normal feature of the metabolism of senescent leaves, associated with the translocation of nitrogen.

The metabolism of proteins during germination and embryonic development of barley seedlings may be regarded as a reversal of the changes outlined above. Experiments have shown that there is a rapid transfer of nitrogen from the endosperm to the embryo during germination under controlled conditions in the light, so that after 6–7 days the reserve is largely converted into tissue protein of the vegetative parts. There is already some direct evidence that the constituent amino-acids of embryonic and endospermic proteins are distinctive. Osborne (1909), in his survey of the cereal proteins, separated an albumin, leucosin, from wheat, barley and other grains. He showed that it was derived mainly from the embryo and contained much smaller amounts of glutamic acid and amide nitrogen than the typical reserve proteins of the endosperm. More recently Bishop (1928) has shown that hordein, with its high content of glutamine, was rapidly converted during malting of the grain.

In the light of this evidence and the data now available concerning the leaf proteins of barley, it seems highly probable that the transfer of nitrogen to the developing embryo involves an extensive reconversion of glutamine into other amino-acids as the preliminary step in the anabolism of tissue proteins. It is noteworthy that Albaum & Cohen (1943) have prepared a highly active transaminase from oat seedlings, where the composition of the reserve proteins also indicates that a rapid conversion of glutamic acid accompanies the formation of proteins in the embryo. Experiments are in progress in this laboratory in order to collect some more exact data concerning the reserve proteins and their relation to the metabolism of the seedlings.

The researches of Hoagland (1944) suggest that a synthesis of glutamine may occur at an early stage in the assimilation of inorganic nitrogen by barley. They have found that excised roots accumulate considerable quantities of this amide when supplied with culture solution containing either nitrates or ammonium salts under appropriate conditions. From this, and other evidence considered in this section, it may be inferred that a substantial proportion of the nitrogen, passing to the active centres of protein synthesis in the seedling and growing plant, is in the form of glutamine.

IV. GLUTAMINE AND THE RESPIRATORY MECHANISM

In an earlier discussion of the respiration of barley leaves, attention was drawn to the close relationship between glutamine and the production of carbon dioxide during the later phase of starvation (Yemm, 1937). There was at that time some evidence to show that glutamic acid, as distinct from almost all other amino-acids, could be oxidized readily in many living tissues. But more recently the study of transaminase systems and the reactions promoted by the specific glutamic acid dehydrogenase has greatly extended our knowledge of the ways in which this amino-acid may participate in the metabolism of the cell.

The origins of carbon dioxide in the respiration of highly starved leaves are undoubtedly complex; carbohydrates probably make a gradually diminishing contribution to the respiratory substrate and are largely replaced by proteins. In absence of an exhaustive analysis of the possible substrates, it is often difficult to detect the relations which may exist between the different components of the system and the total output of carbon dioxide. Nevertheless, many of the experimental records now available demonstrate that fluctuations in the amount of glutamine in the leaves are often closely correlated with changes in the rate of respiration during the later phases of starvation, when the leaf proteins are probably the major source of respiratory substrate. Data collected in three experiments carried out in 1934 and 1939 are set out in Fig. 5 in order to illustrate this feature.

In each of these experiments there was evidence of a close correlation between glutamine content and rate of respiration, extending over a prolonged period of starvation (48–144 hr.) and covering wide fluctuations in the rate of carbon dioxide production. Similar experiments made between 1935–9 have provided eleven records which can be analysed in this way; a direct relation between respiration and glutamine over prolonged periods of starvation is detectable in all but four of the experiments. There are indications that the relation is obscured in leaves having a very high concentration of carbohydrates at the time of detachment. As already noted, it is highly probable that the breakdown of residual carbohydrates may persist until a late phase of starvation. It is, however, consistently observed in all the experiments, that when a high rate of carbon dioxide from protein substrate may be inferred, it is associated with the presence of relatively large amounts of glutamine in the leaves.

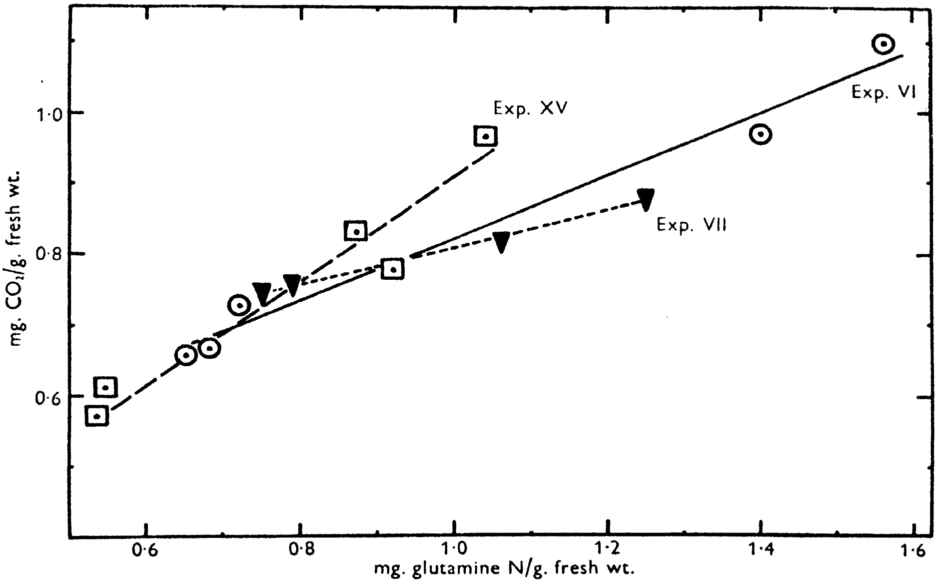
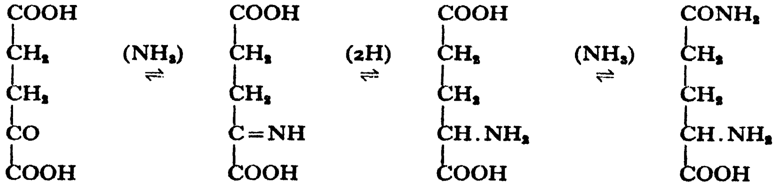


Fig. 5. Relation between the rate of carbon dioxide production and the concentration of glutamine in highly starved leaves (48–144 hr. starvation). Exp. VI (1934), —⊙—; Exp. VII (1934) - - - ▼ - - ; Exp. XV (1939), —⊡—.

There is strong evidence that an important pathway for the metabolism of glutamine is provided by the reversible action of glutamic acid dehydrogenase. Adler, Das, Euler & Heyman (1938) and Adler, Euler, Gunther & Plass (1939) have shown the wide distribution of the enzyme in higher plants, and its linkage with other dehydrogenase systems by means of coenzyme I (Co I). The comprehensive analytical and feeding experiments of Chibnall

(1939) have established beyond reasonable doubt that a synthesis of glutamine from ammonia and α-ketoglutaric acid occurs in grass leaves.



$$\begin{array}{c}COOH\\|\\CH_2\\|\\CH_2\\|\\CO\\|\\COOH\end{array} \overset{(NH_3)}{\rightleftharpoons} \begin{array}{c}COOH\\|\\CH_2\\|\\CH_2\\|\\C{=}NH\\|\\COOH\end{array} \overset{(2H)}{\rightleftharpoons} \begin{array}{c}COOH\\|\\CH_2\\|\\CH_2\\|\\CH.NH_2\\|\\COOH\end{array} \overset{(NH_3)}{\rightleftharpoons} \begin{array}{c}CONH_2\\|\\CH_2\\|\\CH_2\\|\\CH.NH_2\\|\\COOH\end{array}$$

α-ketoglutaric acid α-imino-glutaric acid glutamic acid glutamine

The possibility that these reactions constitute a powerful link between the metabolism of carbohydrates and proteins in plant and animal tissues has been fully discussed by Chibnall (1939), Krebs (1943) and Steward & Street (1947). The hypotheses which have been developed have generally assigned a central and key position to α-ketoglutaric acid, as a convergent point in the metabolism of carbohydrates and proteins in relation to respiratory processes. Many of the changes which occur in the predominantly catabolic systems of detached barley leaves are open to a similar interpretation.

Evidence has been given elsewhere (Yemm & Somers, 1948) that organic acids play an active part in the metabolism of the leaves and several features of the changes of citric, malic and other organic acids are consistent with the view that an organic-acid cycle operates with α-ketoglutaric acid as an intermediary in the conversion of tricarboxylic to dicarboxylic acids. With regard to the mechanism of the breakdown of amino-acids in plants, little is yet known. There are indications, however, that a number of important amino-acids may be converted to α-ketoglutaric acid and glutamic acid in animal tissues. As reviewed by Krebs (1943) the amino-acids, aspartic acid, alanine, arginine, histidine, proline, hydroxyproline, citrulline and possibly lysine may contribute to the formation of glutamic acid and glutamine either by transamination or other reactions. If similar changes occur during the catabolism of leaf proteins, it may be estimated that about half of the tissue protein would enter into oxidative breakdown leading to one or other of the reactants given above.

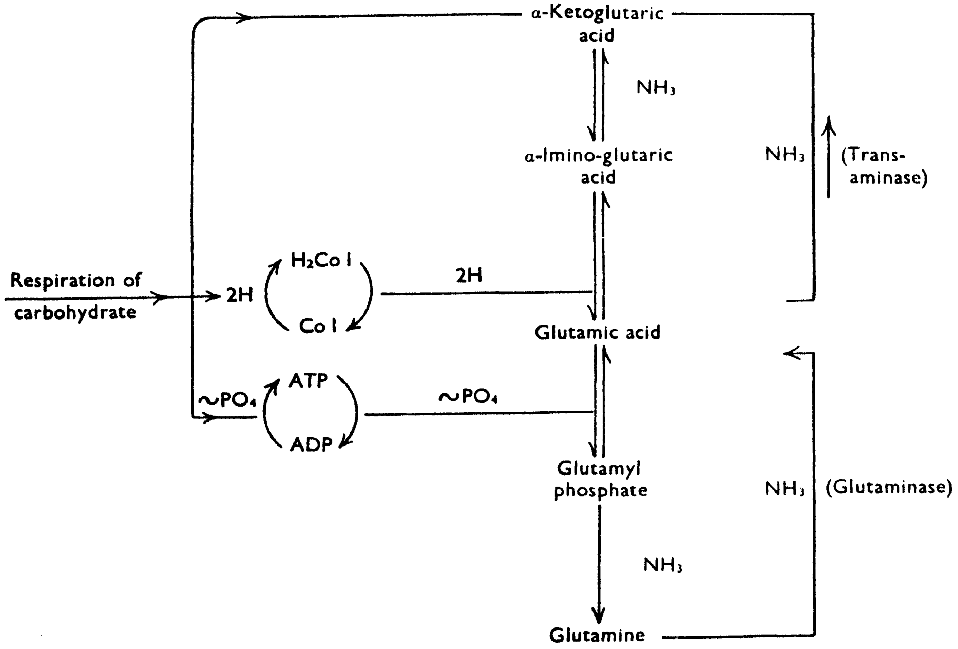
It is probable, therefore, that α-ketoglutaric acid is formed extensively in the leaves during the catabolism of both carbohydrate and protein, and may be an important component of the dynamic equilibria which control the metabolism of the cells. This hypothesis accounts for the interrelations between carbohydrates, respiration, and the synthesis of glutamine during protein breakdown. The rapid formation of the amide in illuminated leaves and at high carbohydrate concentration suggests that under these conditions the catabolism of protein is arrested at this stage by the active production of α-ketoglutaric acid from carbohydrate. Similarly, when the tissues are starved, the rate of formation of α-ketoglutaric acid from either carbohydrate or protein may be an important factor limiting both the production of carbon dioxide and the level at which glutamine is maintained in the cells. It is clear that a study of the influence of α-ketoglutaric acid on the respiration and formation of glutamine in barley leaves is necessary to gain more certain information in this direction.

There is also strong evidence that the formation of glutamine in barley roots is very closely linked with the breakdown of carbohydrate and respiration. As already noted, Hoagland (1944) has found that the amide is rapidly formed during the assimilation of nitrogen by excised roots: in a brief description of his experiments he states that a marked acceleration in the rate of carbon dioxide production and loss of carbohydrate accompanies the synthesis. These observations have been fully confirmed by Mr A. J. Willis, working in this laboratory, and an attempt is being made to analyse the mechanism more completely.

The respiratory and other changes associated with the formation of glutamine in the roots offer a close parallel to some observations recorded by Krebs & Cohen (1939) with animal tissues. They have shown that ammonium salts increase catalytically the production of carbon dioxide, and have obtained evidence that this was associated with the reductive amination of α-ketoglutaric acid to glutamic acid as follows:

$$2\ \alpha\text{-ketoglutaric acid} + NH_3 \rightarrow 1\ \text{succinic acid} + 1\ \text{glutamic acid} + CO_2.$$

Krebs & Cohen suggested that the synthesis of glutamic acid constitutes an important accessory hydrogen-carrier system of the cell, presumably operating with other oxidation-reduction changes through coenzyme I. The oxidative decarboxylation of α-ketoglutaric acid to succinic acid is now generally regarded as an important step in the oxidation of carbohydrate by means of the tricarboxylic acid cycle: the possibility that this step of the cycle may be promoted by linkage with the synthesis of glutamic acid is of great interest with regard to the wider problems of the co-ordination of carbohydrate and protein metabolism in the cell.



The formation of the amide bond of glutamine may also be dependent on the respiratory breakdown of carbohydrate. Recently Speck (1947), Elliot (1948) and Elliot & Gale (1948) have found that the synthesis from glutamic acid and ammonia is brought about by the addition of adenosinetriphosphate (ATP.) to enzyme preparations from animal tissues and micro-organisms. An equimolecular release of phosphate and the positive test for acyl phosphate indicate that glutamyl phosphate is an intermediary in the synthesis.

On the evidence presented here a more comprehensive hypothesis of the cell mechanisms linking the respiratory breakdown of carbohydrate and glutamine synthesis is outlined on p. 327.

The formation of glutamine may thus be co-ordinated with the breakdown of carbohydrate, not only by way of the organic-acid precursor, but also by the catalytic mechanisms of oxidation reduction and phosphorylation. It is likely that linkages of similar nature are engaged in other phases of protein metabolism. An extensive interchange of organic acids between carbohydrates and nitrogenous constituents is highly probable, especially during the synthesis or interconversion of amino-acids; some evidence has already been gained from the study of seedlings and young leaves that the highest rates of respiration are associated with these conditions. Similarly, the participation of high-energy phosphate in the synthesis of the amide, itself effectively an unsubstituted peptide, clearly gives support to the suggestions, originally made by Lipmann (1941), that peptide bonds of the protein may be formed in this way. It is perhaps in its energy relations with respiration that the wide physiological significance of glutamine will be more fully comprehended: the labile amide bond may be a mechanism for the translation of energy, promoting the synthesis of other nitrogenous constituents of the cell, as discussed by Leuthardt (1938).

V. CONCLUSIONS

There is little doubt that glutamine plays a much more conspicuous part than asparagine in the normal metabolism of barley plants. All the available evidence shows that the more stable amide is only formed in large quantities under conditions of acute shortage of carbohydrate, such as are not likely to obtain during growth and development (Archbold, 1942). The intensive catabolism of protein in respiratory processes, often associated with a rise in the free ammonia in the cells, seems to be the special condition leading to the accumulation of asparagine. Whether the role of the two amides may be reversed in other plants, is, at present, an open question; but there are indications that the concentration of readily available carbohydrate is often an important factor influencing their formation in plants. In many tissues where a synthesis of glutamine has been found, such as sugar beet (Vickery, Pucher & Clark, 1936), potato (Steward & Street, 1946), grass leaves and plants (Chibnall, 1939; Greenhill & Chibnall, 1934; Curtis, 1944), a plentiful supply of carbohydrate may be inferred. By contrast, an accumulation of asparagine more commonly occurs in etiolated seedlings and starved leaves. The investigations of Mothes (1940) seem to point in a similar direction. In any case, it is probable that a recognition of the different roles of the amides may help to reconcile some of the conflicting views which have been held with regard to their general significance in plants. For example, in barley plants the relations of asparagine recall the 'detoxification hypothesis' of Prianischnikow (1922), while glutamine demands the wider views of Schultze (1911) on the functions of amides.

A satisfactory interpretation of the role of the amides will only be possible when more is known of the cell mechanism of protein synthesis. This problem has been reviewed by Petrie (1943), Wood (1945) and Steward & Street (1947); no full discussion can be usefully undertaken here. In relation to the metabolism of the amides the work of Schoenheimer

(1942) and Vickery, Pucher, Schoenheimer & Rittenberg (1940) with isotopic nitrogen (N_{15}) is of particular importance. Not only has it shown that the structural proteins of the cell are continuously engaged in dynamic equilibria, but also that glutamic acid, aspartic acid and amide nitrogen take a very active part in these changes. From the energy relations of peptide synthesis it is unlikely that proteolysis is a simple reversible equilibrium, and the conception of a continuous protein cycle, in which catabolism and regeneration are independently controlled (Mothes, 1933; Gregory & Sen, 1937), has received valuable support from the work with isotopic nitrogen. As a result of their investigations of potato tissue, Steward & Street (1946, 1947) have suggested that glutamine occupies a key position in the synthesis of protein, and have fully discussed the potentialities of the amide from this point of view. The metabolism of the tissue is tentatively interpreted in terms of interlocking cycles of proteins and organic acids. This general hypothesis would readily account for many of the interrelations between glutamine and protein metabolism so far revealed by work with barley plants. It is likely that further study of the amides of the normal growing plant, and their fluctuations under the varied conditions of protein synthesis and breakdown in young and senescent tissues, will provide more decisive evidence of the role of the amides.

VI. SUMMARY

Evidence, assembled in this paper, indicates that glutamine plays an important part in the metabolism of nitrogen during the normal growth and development of barley plants. The experimental data which are considered from this point of view may be summarized as follows:

1. Glutamine is readily formed as a result of secondary breakdown of tissue protein in senescent leaves rich in carbohydrates.
2. The amide is an important component of the reserve proteins in the grain, and the changes of nitrogenous constituents during ripening and germination suggest that the amide occupies a key position in the interconversion of amino-acids.
3. Under suitable conditions barley roots elaborate the amide rapidly when they assimilate nitrates or ammonia. It is probable that a substantial proportion of the nitrogen reaching the meristematic parts of the plant, or other centres active in protein synthesis, is in the form of glutamine.

There is evidence that the amide is one of the important links between the metabolism of carbohydrates and proteins; the cell mechanisms engaged in the synthesis of glutamine are considered in this regard. Both from the nature of the precursor, α-ketoglutaric acid, and the enzyme systems of oxidation reduction and phosphorylation, the formation of glutamine may be closely integrated with the breakdown of carbohydrate and cell respiration. It is concluded that these interlinked mechanisms are important in relation to the wider problem of the co-ordination of the metabolism of carbohydrate and proteins in the plant.

The author wishes to thank Prof. Macgregor Skene for his helpful criticism of this paper.

REFERENCES

Adler, E., Das, N. B., Euler, H. V. & Heyman, U. (1938). Biologische Dehydriering und Synthese der Glutaminsaüre. *C.R. trav. lab. Carlsberg*, **22**, 15–24.

Adler, E., Euler, V. H., Gunther, G. & Plass, M. (1939). Isocitric dehydrogenase and glutamic acid synthesis in animal tissues. *Biochem. J.* **33**, 1028–45.

Albaum, H. G. & Cohen, P. P. (1943). Transamination and protein synthesis in germinating oat seedlings. *J. Biol. Chem.* **149**, 19–27.

Archbold, H. K. & Mukerjee, B. N. (1942). Physiological studies in plant nutrition. XII. Carbohydrate changes in the several organs of barley plant during growth, with special reference to the development and ripening of the ear. *Ann. Bot.* N.S. **6**, 1–41.

Archibald, R. M. (1945). Chemical characteristics and physiological roles of glutamine. *Chem. Rev.* **37**, 161–208.

Bishop, L. R. (1928). The composition and quantitative estimation of barley proteins. I. *J. Inst. Brew.* **34**, no. 3, 101.

Chibnall, A. C. (1939). *Protein Metabolism in the Plant*. New Haven.

Csonka, F. A. & Jones, D. B. (1929). The glutelins of rye and barley. *J. Biol. Chem.* **82**, 17.

Curtis, L. C. (1944). Exudation of glutamine from lawn grass. *Plant Physiol.* **19**, 1–5.

Damodaran, M. (1932*a*). The isolation of asparagine from an enzymic digest of edestin. *Biochem. J.* **26**, 235–47.

Damodaran, M., Jaaback, G. & Chibnall, A. C. (1932*b*). The isolation of glutamine from an enzymic digest of gliadin. *Biochem. J.* **26**, 1704–13.

Elliott, W. H. (1948). Adenosine triphosphate in glutamine synthesis. *Nature, Lond.*, **161**, 128.

Elliott, W. H. & Gale, E. F. (1948). Glutamine-synthesising system of Staphylococcus aureus: its inhibition by crystal violet and methionine-sulphoxide. *Nature, Lond.*, **161**, 129.

Greenhill, A. W. & Chibnall, A. C. (1934). Exudation of glutamine from perennial rye-grass. *Biochem. J.* **28**, 1427.

Gregory, F. G. & Sen, P. K. (1937). Physiological studies in plant nutrition. VI. The relation of respiration rate to carbohydrate and nitrogen metabolism of the barley leaf as determined by nitrogen and potassium deficiency. *Ann. Bot.* N.S. **1**, 521–61.

Hoagland, R. D. (1944). *Lectures in the Inorganic Nutrition of Plants*. Waltham, Massachusetts: Chronica Botanica.

Krebs, H. A. (1943). The intermediary stages in the biological oxidation of carbohydrates. *Advances in Enzymology*, **3**, 191–252.

Krebs, H. A. & Cohen, P. P. (1939). The metabolism of α-ketoglutarate in animal tissues. *Biochem. J.* **33**, 1895.

Lamour, R. K. (1927). A comparative study of the glutelins of cereal grains. *J. Agric. Res.*

Leuthardt, F. (1938). Untersuchungen über die Harnstoffbildung aus Glutamin. *Z. physiol. Chem.* **252**, 238.

Lipmann, F. (1941). Metabolic generation and utilization of phosphate bond energy. *Advances in Enzymology*, **1**, 99–162.

Mothes, K. (1933). Die naturliche Regulation des pflanzenlichen Eiweisstoffwechsels. *Ber. dtsch. bot Ges.* **51**, 31–46.

Mothes, K. (1933*b*). Sauerstoffpotential und Eiweissumsatz im Laubblatt. *Flora*, **128**, 58.

Mothes, K. (1940). Quoted by Steward & Street (1947).

Osborne, T. B. (1909). *The Vegetable Proteins*. London.

Paech, K. (1935). Über die Regulation des Eiweissumsatzes und über den Zustand der proteolytischen Fermente in den Pflanzen. *Planta*, **24**, 78.

Petrie, A. H. K. (1943). Protein synthesis in plants. *Biol. Rev.* **18**, 105–8.

Prianischnikow, D. (1922). Über den Aufbau und Abbau des Asparagins in den Pflanzen. *Ber. dtsch. bot. Ges.* **40**, 242–8.

Richards, F. J. & Templeman, W. G. (1936). Physiological studies in plant nutrition. IV. Nitrogen metabolism in relation to nutrient deficiency and age in leaves of barley. *Ann. Bot.* **50**, 367–401.

Schoenheimer, R. (1942). *The Dynamic State of Body Constituents*. Harvard Press.

Schultze, E. (1911). *Z. Physiol. Chem.* **65**, 431.

Schwab, G. (1936). Studien über Verbreitung und Bildung der Saüreamide in der hoheren Pflanzen. *Planta*, **25**, 579–606.

Speck, J. F. (1947). The enzyme synthesis of glutamine. *J. Biol. Chem.* **168**, 403.

Steward, F. C. & Street, H. E. (1946). The soluble nitrogen fractions of potato tuber, the amides. *Plant Physiol.* **21**, 155–93.

Steward, F. C. & Street, H. E. (1947). The nitrogenous constituents of plants. *Ann. Rev. Biochem.* **16**, 471–502.

Vickery, H. B. & Pucher, G. W. (1939). The metabolism of amides in green plants. III. The mechanism of amide synthesis. *J. Biol. Chem.* **128**, 703–13.

Vickery, H. B., Pucher, G. W. & Clark, H. E. (1936). Glutamine metabolism of the beet. *Plant Physiol.* **11**, 413–22.

Vickery, H. B., Pucher, G. W., Schoenheimer, R. & Rittenberg, D. (1940). The assimilation of ammonia nitrogen by the tobacco plants; a preliminary study with isotopic nitrogen. *J. Biol. Chem.* **135**, 531–9.

Wood, J. G. (1945). Nitrogenous constituents of plants. *Ann. Rev. Biochem.* **14**, 665–84.

Wood, J. G., Cruickshank, D. H. & Kuchel, R. H. (1943). The metabolism of starving leaves 1, 2 and 3. *Aust. J. Exp. Biol. Med. Sci.* **21**, 37–53.

Wood, J. G., Mercer, F. V. & Pedlow, C. (1944). The metabolism of starving leaves 4. *Aust. J. Exp. Biol. Med. Sci.* **22**, 38–43.

Wood, J. G. & Cruickshank, D. H. (1944). Metabolism of starving leaves 5. *Aust. J. Exp. Biol. Med. Sci.* **22**, 111–23.

Yemm, E. W. (1935). Respiration of barley plants. II. Carbohydrate concentration and carbon dioxide production in starving leaves. *Proc. Roy. Soc.* B, **117**, 504–25.

Yemm, E. W. (1937). Respiration of barley plants. III. Protein catabolism in starving leaves. *Proc. Roy. Soc.* B, **123**, 243–73.

Yemm, E. W. (1948). Respiration of barley plants. V. Protein catabolism and the formation of amides in starving leaves. (In the Press.)

Yemm, E. W. & Somers, G. F. (1948). Respiration of barley plants. IV. The metabolism of organic acids in starving leaves. (In the Press.)

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THE ANNONACEOUS SEED AND ITS FOUR INTEGUMENTS

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(With 31 figures in the text)

CONTENTS

	PAGE
Introduction	332
Annonaceous seed characters	333
New terms	333
Abbreviations	335
Method	335
Anaxagorea javanica	336
Annona squamosa	340
Desmos dasymaschala	344
D. chinensis and *D. dumosa*	346
Xylopia fusca, *X. caudata* and *X. malayana*	347
Polyalthia purpurea	347
Artabotrys odorata	349
Popowia	351
Alphonsea elliptica	351
Canangium odoratum	352
Mezzettia leptopoda	358
Xylopia Curtisii	358
Morphological principles	360
Postponed development	360
Transference of function	360
Organizational centres	362
Classification	362
Middle integument	363
Summary	363

INTRODUCTION

In 1930 I began, in the Singapore Botanical Garden, the study of the seeds of tropical plants, and I continued this work until 1942 by collecting and preserving the fruits which I picked up on the floor of the forest, and by drawing the stages of germination of their seeds. During the war years, 1943–5, when the Botanical Garden was under the charge of Dr Kwan Koriba, of Kyoto University, I started a more detailed study of the microscopic structure of the seeds in many families of tropical trees. I am greatly indebted not only to Dr Koriba for having enabled me to continue my researches while an 'enemy alien', but also to Prof. Hidezo Tanakadate, of Sendai University, who took the initiative in 1942 in preserving the collections of the Botanical Garden on the military seizure of Singapore. Many circumstances have since interrupted this research, which had begun to reveal a variety of problems that I had hoped to pursue, but it seems preferable to publish what I have completed without further delay, especially as the subject should grow rapidly in many directions.

I have chosen the Annonaceae as an introduction principally because their large seeds illustrate the complexity of structure which has to be considered in the morphology of tropical seeds and which is scarcely to be gathered from microspermous temperate floras, as witnessed by the facility with which Annonaceae are generally related to Ranunculaceae. Secondly, this complexity helps to reveal certain physiological processes which seem but vaguely to be comprehended in text-books, yet without which one can have little appreciation of the mechanism of structural variation in multicellular plants. These processes are:

(1) Postponed development, particularly the delay from prefertilization to postfertilization.

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(2) Transference of function, whether from organ to organ, tissue to tissue, cell-layer to cell-layer or cell to cell.

(3) Organizational centres, as seats of tissue differentiation (possibly of hormone production).

In addition, there is the importance of the microscopic structure of the seed in classification, and there is the Annonaceous peculiarity of multiple integuments.

The specific details recorded in the main body of the paper are a category of facts for future elaboration. It will be sufficient to read the introductory sections, to study the illustration, and to pass to the final section, in order to perceive the great interest of seed structure.

ANNONACEOUS SEED CHARACTERS

So far as I have discovered, the Annonaceous seed and ovule have the following peculiarities:

(1) The anatropous ovule is either erect with the micropyle pointing to the base of the carpel or transverse, as in many-seeded carpels.

(2) The inner integument commonly exceeds the outer so as to give a prominent naked endostome to the ovule, persistent even in the seed.

(3) The chalaza of the ovule and seed is uncondensed, so that I describe them as *perichalazal* (see below). The character is caused by the pronounced basipetal growth of the ovule and developing seed at the end opposite to the micropyle.

(4) The nucellus develops greatly, after fertilization, so as to fill the entire, full-grown, but immature seed.

(5) The soft nucellus becomes ruminate by infoldings of the integuments on the sides of the seed, the ruminations being mostly transverse.

(6) The endosperm develops in the form of a longitudinal, centrally placed, cigar extending from the micropyle to the chalazal end, as the seed grows basipetally. Eventually, it expands into the nucellus between the ruminations (which are initially a feature of the nucellus before the endosperm has attained any appreciable size).

(7) The inner integument does not thicken and, except for forming the endostome, generally disappears from the mature seed.

(8) The funicle is very short and the seeds are practically sessile.

(9) The hilum is ill-defined because the exostome is more or less lacking between the funicle and endostome: commonly the naked endostome appears as a minute micropyle. As a result, the hilum is usually closed internally by a plug of woody tissue round the endostome (the deficiency of the hilum doubtless contributing to the small power of dormancy of the seeds, by facilitating desiccation through the broken tissue of the hilum).

(10) The testa is fibrous, consisting of longitudinal and oblique fibres.

(11) The embryo is small and develops late (only after the seed is almost full-grown).

These characters introduce a new set of comparisons between the Annonaceae, Lauraceae, Myristicaceae, Magnoliaceae and other related families.

NEW TERMS

Transmedian. A median longitudinal section cuts a bilaterally symmetrical body into similar halves. A longitudinal section at right angles to the median cuts the body into dissimilar halves, variously called dorsal and ventral, posterior and anterior, or abaxial and adaxial. I call such a section the transmedian longitudinal because it passes through the apex and base of the body at right angles to the median, the term *translongitudinal* being required for any section in a plane parallel to the transmedian longitudinal. The term is necessary to describe sections of the anatropous ovule taken through the micropyle and chalaza (as apex and base) at right angles to the median longitudinal which divides the raphe in similar halves, cf. Fig. 9A, B.

Perichalazal. I use this name to describe one of the main features of the Annonaceous ovule (or seed). In the ordinary anatropous ovule, the chalaza lies at the opposite end to the micropyle, and the inner integument is free from the nucellus and outer integument throughout its length. The chalaza is, thus, at the point of inflexion of the anatropous ovule and is the small region, basal to the ovule proper, where the integuments and nucellus are joined to the funicle and where, commonly (but by no means always), the vascular bundle of the raphe terminates by transfusion to the nucellus. In the mature Annonaceous ovule, as in the seed, this is not the case. The vascular bundle extends as a hoop in the median longitudinal plane from the funicle almost to the micropyle, and both the integuments and the nucellus are joined to the ground tissue of the vascular bundle along the whole of its course, being free only on the sides of the ovule, that is on the parts which flank the vascular hoop. But there is an exception either in the region of the micropyle or in the distal half of the ovule, where both integuments and nucellus are completely free; and in the seed, this is the case only in the relatively microscopic region of the micropyle. The explanation lies in the *basipetal* growth of ovule and seed. When the ovuliferous rudiment changes its direction of growth to form the anatropous body of the ovule, the two integuments begin to arise but, very soon, the growth of the ovule continues mainly in the narrow zone between the circles of origin of the integuments and the reflexed apex of the funicle. This is the chalaza of the normal anatropous ovule and the effect of its basipetal growth is that the normal body of the ovule with free integuments, is carried away from the apex of the funicle and remains in a rudimentary condition round the micropyle, while the chalaza expands into the body of the ovule round which the vascular bundle is extended as a hoop. The basipetal direction of growth continues after fertilization so that the expanded chalaza is dilated still further to form the body of the seed, and the original body of the ovule, with completely free integuments is limited microscopically to the micropylar region, cf. Figs. 6H, 23C, 29A, B. On the sides of the ovule and of the seed, however, the integuments and nucellus are free. Hence, the peculiar feature is the basipetal growth which extends the chalazal region in the medium plane round the seed, for which reason I have called it *perichalazal*.

The following are the consequences of perichalazal growth:

(1) The vascular bundle of the raphe extends round the seed.

(2) The integuments and nucellus are connected with the raphe through its length.

(3) The micropyle is thrust further from the base of the seed and, consequently, the endosperm grows into the large nucellus as a cigar-shaped body extending from the micropyle to the opposite end, or chalazal base, of the ovule.

(4) The ruminations, developing from the parts of the integuments which are free to fold, arise from the sides of the seed, and not from the vascular hoop, so that the median longitudinal section of the seed shows the endosperm free of ruminations.

(5) The ruminations are transverse folds caused by overgrowth of the integuments on the flanks of the rigid vascular hoop and proceeding basipetally as the seed enlarges.

(6) The exostome is little developed because its growth soon ceases, and thus it seldom

(perhaps never) separates the hilum from the endostome which is often visible at the base of the mature seed.

Antiraphe. The chalaza of the ordinary anatropous ovule and the chalazal base, where the nucellus and integuments are attached to the raphe in the transmedian longitudinal section of the Annonaceous ovule, mark the limitation of the raphe, or stalk, of the ovule. The opposite side of the vascular hoop to the raphe I refer to as the *antiraphe*, cf. Figs. 23, 26A and 31A, as *rp.* and *arp.* respectively.

Arilostome. In the seed of *Annona*, a rudimentary aril surrounds the hilum and micropyle to form an apparent micropyle, which I call the *arilostome*, in comparison with the deficient exostome and the minute endostome.

Subhilar tissue. I regard the hilum as the scar where the seed breaks from the funicle or placenta. The tissue between this superficial scar and the endosperm of the seed I call the *subhilar tissue*. It is well developed, often as a conical woody block or plug in Annonaceous seeds, possibly as the mechanical substitute for the deficient exostome on the raphe side of the endostome. The woody plug closes the gap which is thus left in the testa on the detachment of the seed.

Middle integument. I use this term for the tissue which develops on the sides of the ovule between the outer and inner integuments and which forms the main part of the testa in some Annonaceous seeds, as *Canangium odoratum*, *Mezzettia* and *Xylopia* spp.

ABBREVIATIONS IN FIGURES

In the Figures I have used the following recurrent abbreviations without explanation in the legends:

ar.	aril	*i.e.*	outer integument
c.o.	oil-cells	*i.i.*	inner integument
cu.	cuticle	*i.m.*	middle integument
em.	embryo	*m.*	micropyle
en.	endosperm	*n.*	nucellus
h.	hilum	*v.b.*	vascular bundle

I have also used the abbreviations *i.e.*, *i.i.*, *i.m.* and *v.b.* in the text.

METHOD

I have used, wherever possible, fresh-living tissue. I have dissected under the binocular microscope, then cut sections under the binocular microscope in order to see exactly the orientation of the section (as well as to secure the thinnest possible section, without the need of the microtome), and after examination of the living material, I have cleared the sections in dilute potash in order to make the microscopic drawings. It is generally impossible to make out the microscopic structure of the mature seed, especially the derivation of the cell-layers of the testa, because of the hardness of parts of the testa and the collapse, or shrivelling, of the soft parts. The structure of the testa is best seen in full-grown, but immature seeds shortly before the seed begins to dry off and harden. But, in all cases, in order to be sure of the derivation of the cell-layers, it is necessary to trace all stages of the development of the seed from the ovule. In this connexion it should be noted that Annonaceous flowers generally open widely long before they are full-grown and ready for pollination: thus, with preserved material only, it is impossible to judge if a flower is full-grown and if the ovule is mature.

Anaxagorea javanica

The simple seeds of this common, small Malayan tree serve well as an introduction. The follicles are capsular and contain two hard, black, shiny seeds, pressed tightly together: the ripe follicles open and then contract, as they dry, until they eject with a click both seeds simultaneously for a considerable distance. In this respect the genus seems to be unique in the family. Whether the comparatively small size of the seed, the simple rumination and the aerenchyma of the inner layer of the testa are primary or derived features, it is difficult to say, but there is no evidence to suppose that they are not primitive.

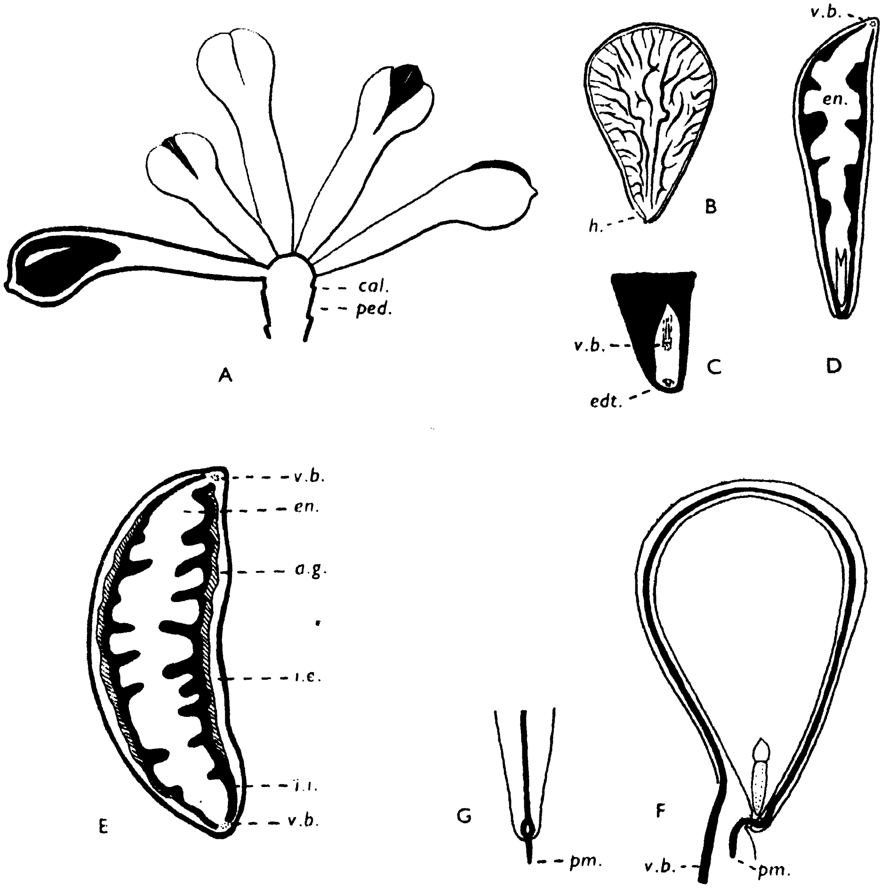

Fig. 1. *Anaxagorea javanica.* A, a cluster of ripe follicles in section, the left-hand follicle in longitudinal section, × 1; B, a seed with the testa removed from one half, to show the ruminations developed from the centre of the flank of the seed outward to the periphery, × 2; C, the hilum, × 5; D, a transmedian longitudinal section of the seed, the ruminations shown in black, × 3; E, a transverse section of a seed, the ruminations shown in black, and the air-gap between *i.e.* and *i.i.* hatched, × 7; F, a median longitudinal section of a seed to show the vascular bundle (in black), × 4; G, the vascular supply of the micropyle, × 4. *a.g.*, air-gap; *cal.*, calyx scar; *edt.*, endostome; *ped.*, pedicel; *pm.*, short, blind post-micropylar vascular bundle.

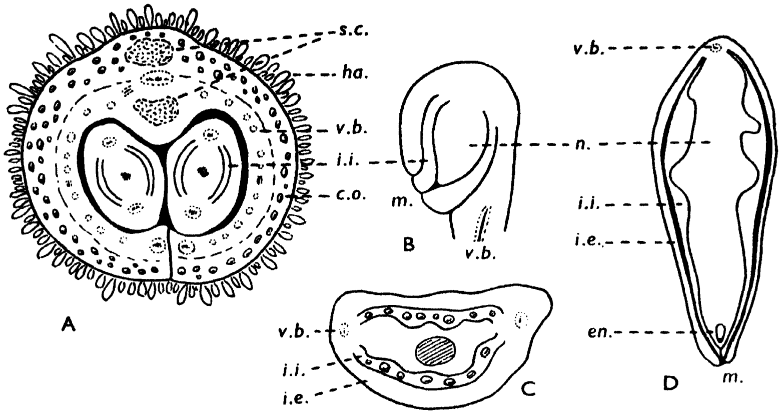

Fig. 2. *Anaxagorea javanica.* A, a transverse section of a carpel of a mature flower, × *c.* 50; B, an oblique, or tangential, longitudinal section of the ovule of a mature flower, × *c.* 50; C, a transverse section of a young seed, half-grown, with the ruminations just beginning to form by folding of *i.i.* (with oil-cells), × 15; D, a transmedian longitudinal section of a half-grown seed, showing the large nucellus, incipient rumination, and rudimentary endosperm, × 7. *ha.*, vesicular clavate hairs of ovary wall; *s.c.*, groups of stone cells external and internal to the dorsal *v.b.* of ovary.

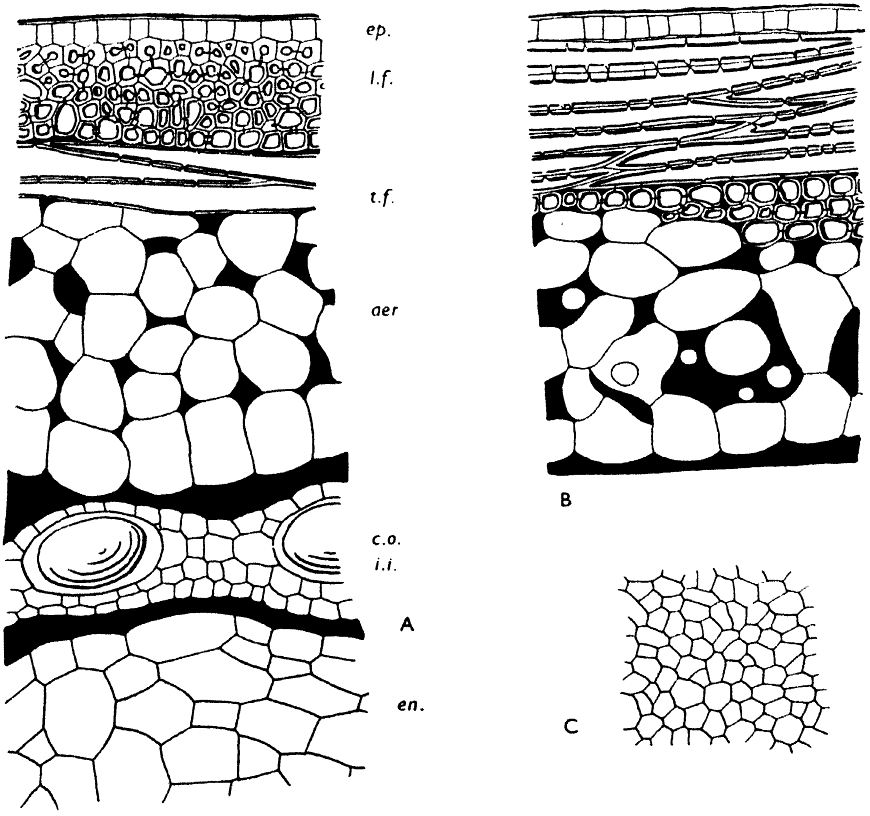

Fig. 3. *Anaxagorea javanica.* A, a transverse section of the testa and outer part of the endosperm of an almost mature seed (the air-spaces shown in black), × 225; B, the testa of the same seed in longitudinal section, × 225; C, the epidermis of the testa in surface view, × 225; *aer.*, aerenchyma; *ep.*, epidermis; *l.f.*, longitudinal fibres; *t.f.*, transverse fibres.

Seeds. Generally two in each follicle, plano-convex with rounded-triangular outline, the hilar end being pointed, *c.* 15 × 9 × 3·5 mm., white, then mottled brown, ripening black, hard, shiny, glabrous, very smooth and slippery; *hilum*: small, oblong, white, composed of the dry powdery aerenchyma of *i.e.*, through which the minute endostome projects (the exostome remaining on the ovary wall); *funicle*: none; *endosperm*: white, with few, short, pale brown ruminations from the broad sides of the seeds.

Testa. Derived from *i.e.* and composed of: (1) an epidermis of well-defined, short, isodiametric cells, thin-walled but becoming filled with dark brown gummy matter, giving the black colour; (2) a thick layer of longitudinal, pitted fibres, the outer ones with brownish walls; (3) a narrow layer, 1–3 cells thick, of transverse or oblique fibres, but incomplete in places; (4) a relatively wide layer of substellate, thin-walled, colourless parenchyma, drying up and leaving an air-gap between the testa and endosperm on the sides of the seed (this layer is thickened at the hilum and forms its friable white tissue).

Inner integument. Composed of 3(–4) cell-layers, always thin-walled, the middle layer of cells containing many enlarged oil-cells (the only oil-cells in the seed-coat): drying into a brownish pellicle.

Ruminations. Formed by the *i.i.* from the flanks of the seed, as shallow folds.

Nucellus. Persistent as a single layer of small, thin-walled, epidermal cells, slightly elongate longitudinally and with thinly cuticularized outer walls.

Endosperm. With thin-walled cells, full of oil-droplets but without starch: the embryo composed of similar tissue.

Structure of ovule. The raphe is continuous round the ovule, except at the micropyle where the *i.i.* is free from the *i.e.* on all sides. This fact is shown in the transverse section of the ovule of the flower before fertilization (Fig. 2A, 2B being an oblique, or tangential, longitudinal section). The *i.i.* is 3 cells thick, the *i.e.* about 5 cells thick; and the large nucellus fills the body of the ovule.

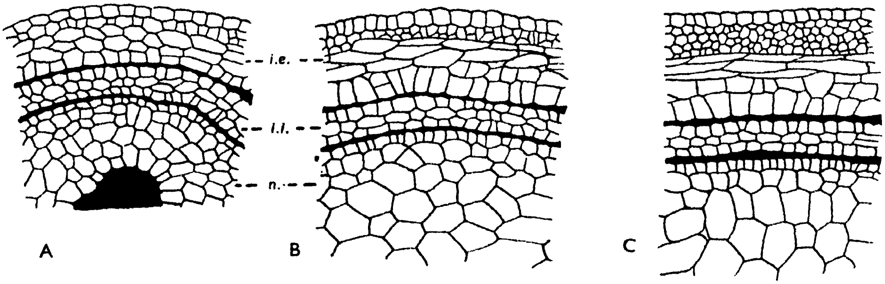

Fig. 4. *Anaxagorea javanica.* Transverse sections of the walls of the ovule and developing seed, × 225; to show the early differentiation of *i.e.* A, the ovule of the mature flower; B, soon after fertilization; C, a little later than B.

Development of the seed. The *i.e.* quickly differentiates into its four layers. The outermost undergoes only anticlinal cell divisions, which are continued until the seed is full-grown, and then this layer becomes the epidermis. The second layer of cells, from the outside, forms the longitudinal fibres by anticlinal and periclinal division, accompanied by sliding growth. The third and fourth layers form the transverse and oblique fibres mainly by anticlinal division and sliding growth. The fifth, innermost, layer slowly forms the aerenchyma of the growing seed, by parenchymatous division of the cells, particularly at the hilum where communication is made with the cortex of the follicle (note the ovule is soon pressed tightly to the ovary wall so that there is no air-gap to respirate the seed).

The *i.i.* does not undergo periclinal division, the cells multiplying by anticlinal division and enlargement.

The nucellus grows rapidly and, when the seed is full-sized, but still immature, it is filled with white, pulpy-firm, nucellar tissue. The endosperm then begins to form and to replace the nucellus, which becomes reduced to its outer layer of cells. As the nucellar cells begin to disintegrate before the advancing endosperm, they enlarge greatly, particularly the cells of the hypodermis (Fig. 5 A).

The ruminations begin to develop, as infoldings of the *i.i.* into the soft nucellus, when the seed is about half-size. The ruminations are completed before the nucellus is replaced by the endosperm.

The embryo develops at a late stage, immediately after the endosperm has substituted the nucellus.

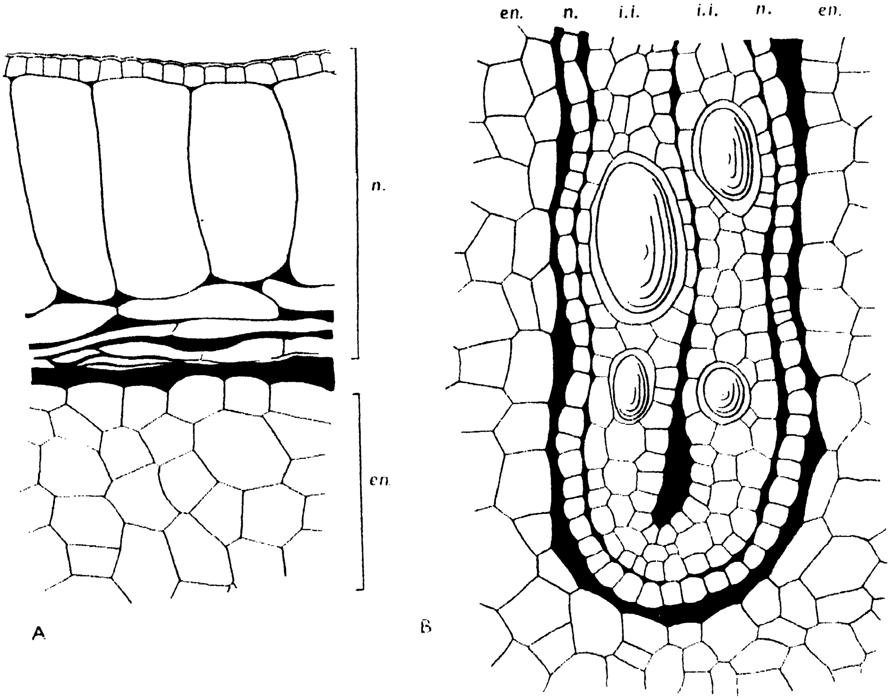

Fig. 5. *Anaxagorea javanica.* A, a transverse section of the edge of the nucellus when most of its tissue has receded before the endosperm, × 225; B, the end of a rumination of a full-grown seed, × 225.

The minute exostome becomes embedded in the base of the ovary, where it breaks off on ripening of the seed. Thus, the endostome projects from the ill-formed hilum.

Vascular supply of the seed. A single, unbranched, vascular bundle extends round the seed (in the peripheral chalaza along the edges of the seed). At the micropyle, this bundle divides into two branches, which form a ring in the *i.e.* round the micropyle, and reunites to pass into the placenta as a short, blind bundle (Fig. 1 F, G). It is, doubtless, this blind end of the vascular bundle which pegs the exostome to the ovary wall. It may represent a vestige of the aril, of other Annonaceous genera.

The vascular supply is defined as an embryonic strand of conducting tissue in the ovule of the mature flower.

Annona squamosa

This is the well-known Custard Apple. The black seeds, one per carpel, are embedded near the periphery of the fleshy receptacular fruit, analogous with a gigantic raspberry. I find that the seeds of *A. muricata*, the Soursop, have essentially the same structure, the most surprising feature being a rudimentary aril. Otherwise, except for being plumper,

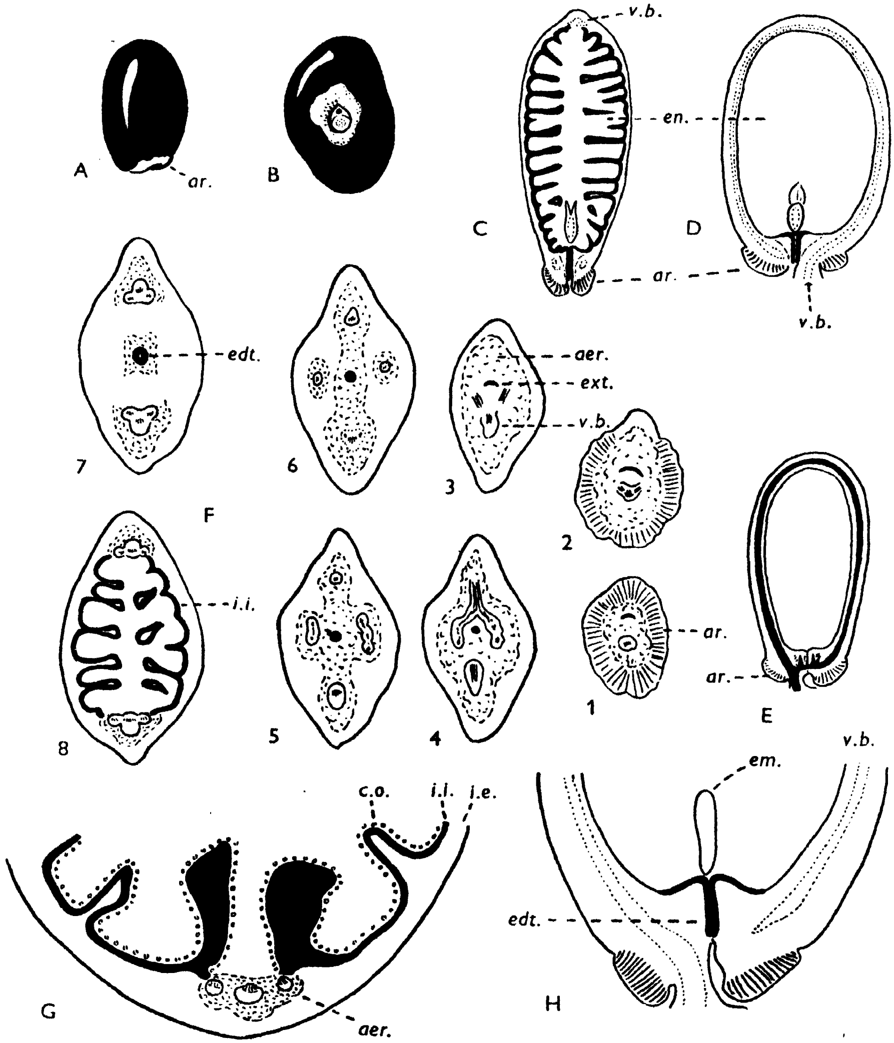

Fig. 6. *Annona squamosa*. A, seed in side view, × 1½; B, hilum end of seed, showing the aril surrounding the hilum, with micropyle and *v.b.*, × 3; C, a transmedian longitudinal section of the seed, the *i.i.* shown in black, the *v.b.* dotted, × 3; D, a median longitudinal section of the seed, the free part of the *i.i.* (forming the endostome) shown as a heavy line, × 3; E, a diagram of the vascular supply of the seed, the *v.b.* in black; F, 1–8, serial transverse sections of the base of the seed to show the vascular supply of the micropylar-subhilar region (1–3 across the exostome, 4–7 across the endostome), × 15; G, a transverse section of the edge of a seed, × 15; H, a median longitudinal section of the base of a full-sized, but immature seed, to show the small free portion of the endostome (heavy line), × 7. *aer.*, aerenchyma; *edt.*, endostome; *ext.*, exostome.

the seeds resemble those of *Anaxagorea* in general appearance. Microscopically, there are differences in the structure of the outer integument and the larger ruminations.

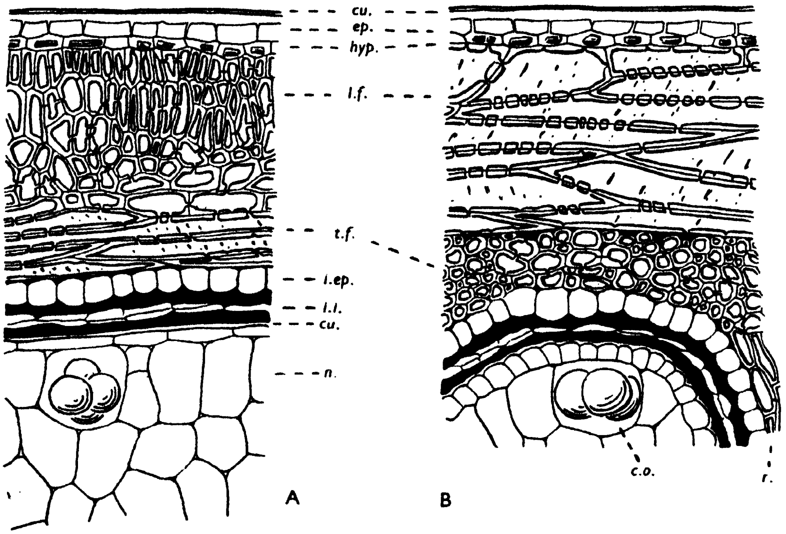

Fig. 7. *Annona squamosa*. Transverse (A) and longitudinal (B) sections of the testa of a full-sized seed, the endosperm as yet not fully developed, × *c.* 225. *ep.*, epidermis; *hyp.*, hypodermis with crystals; *i.ep.*, inner epidermis of *i.e.*; *l.f.*, longitudinal fibres; *r.*, part of a rumination; *t.f.*, transverse fibres.

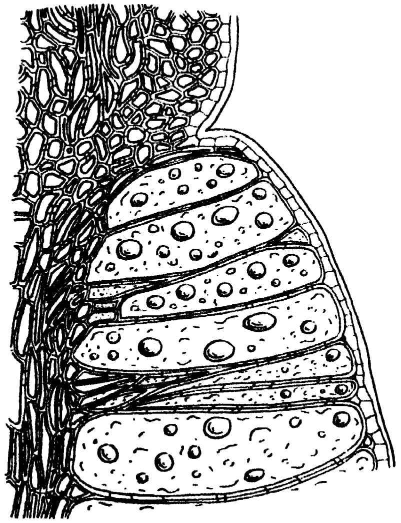

Fig. 8. *Annona squamosa*. A longitudinal section of the mature aril at its junction with the testa, × *c.* 75.

Seeds. *c.* 13 × 7·5 × 5 mm., rounded-oblong, subangular in section, hard, shiny, black, but covered with a thin pulpy membrane; ***aril***: small, white, horny-pulpy, sharply delimited, surrounding the hilum (as an arilostome); ***hilum***: small, white; ***ruminations***: light brown, as slightly flattened pegs (not plates) from the flanks of the seed, the inner ends often somewhat expanded; ***endosperm***: white, horny.

Testa. Derived from *i.e.* and consisting of: (1) an epidermis of isodiametric colourless cells, the outer walls being much thickened, the roof of the cell cavity flat or slightly convex, the other walls scarcely thickened; (2) a hypodermis consisting of a single row of small, thin-walled, somewhat compressed, colourless cells containing a rod or lump of crystalline matter (?calcium oxalate); (3) a layer of longitudinal brown-walled fibres, 3–5 cells thick; (4) a layer of transverse or oblique fibres, 3–5 cells thick; (5) an inner epidermis of thin-walled, colourless, isodiametric cells.

Aril. Composed of a single layer of very large, 0·5–1 mm. long, cells elongated anticlinally, corresponding with the hypodermis of the testa, and covered by an epidermis of small isodiametric cells; the enlarged hypodermal cells with slightly thickened, pitted walls and filled with fine oil-droplets (giving a milky juice on crushing) without free lobes.

Inner integument. Only 2 cells thick, collapsing before the seed is full-grown, often when it is only half-size.

Ruminations. Composed of foldings of the *i.i.* into which extend the inner epidermis and transverse fibres of the *i.e.*

Nucellus. Absent from the mature seed, except for many hypodermal cells (of the nucellus) containing 3–4 large oil-drops (arranged usually in tetrads) and becoming incorporated in the outer layers of the endosperm.

Endosperm. The cells with slightly thickened walls and oily contents (no starch).

Subhilar tissue. Forming a flattened woody cone of thick-walled, isodiametric cells round the endostome, and separated by collapsed aerenchyma from an outer woody layer continuous with the *i.e.*; this aerenchyma continuous along the whole length of the *v.b.* of the testa.

Structure of the ovule. The ovule is constructed as in *Anaxagorea*. The *i.e.* is about 5 cells thick, the *i.i.* only 2 cells thick.

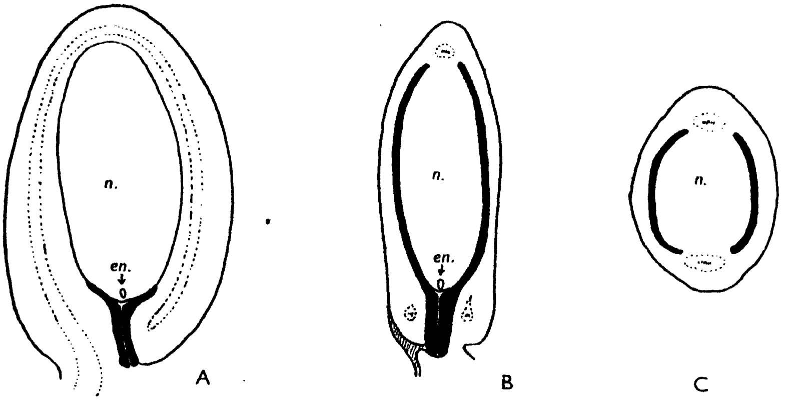

Fig. 9. *Annona squamosa.* Sections of a young seed, *c.* 2 mm. long, as yet without aril, × *c.* 25; A, median longitudinal; B, transmedian longitudinal; C, transverse; the *i.i.* shown as a heavy line, the *v.b.* dotted.

Development of the seed. As in *Anaxagorea*, the *i.e.* quickly develops its tissue layers, while the *i.i.* merely increases in extent without thickening or differentiation. The nucellus fills the whole of the cavity of the full-grown, but immature seed, and many of its hypodermal cells develop the strikingly arranged, large, oil-drops. Later, the endosperm substitutes the whole of the nucellus except for these oil-cells which become embedded in the outer part of the endosperm and appear to be part of its tissue. There are no oil-cells in any other part of the seed. The aril begins to form in seeds about 3–4 mm. long, and develops simply by enlargement, particularly anticlinal, of the hypodermal cells of the *i.e.* round the exostome, which is thus converted into an arilostome, and of the very short funicle: at maturity, the cells immediately internal to the large cells of the aril become thick-walled. (There are no crystal-bearing cells in the aril.) As in *Anaxagorea*, the endosperm develops axially in the nucellus, as a cigar-shape, and then extends outward between the ruminations.

At maturity, the radial walls of the epidermis and hypodermis of the testa dissolve, and these two layers separate, as the slimy, colourless pellicle, from the black, fibrous inner layers of the testa. The walls of the fibres become dark brown: the two fibre layers shrink from each other, and the inner epidermis dries up.

Vascular supply of the seed. The vascular arrangement is essentially the same as in *Anaxagorea*, but the short post-micropylar *v.b.* joins with the *v.b.* of the funicle and, on either side of the micropyle, 2–4 short, subanastomosing, *v.b.* arise from the micropylar ring and end blindly in the thickened tissue of the subhilum. The single *v.b.* surrounding the seed appears tripartite in transverse, microscopic, section (Fig. 6 F, G).

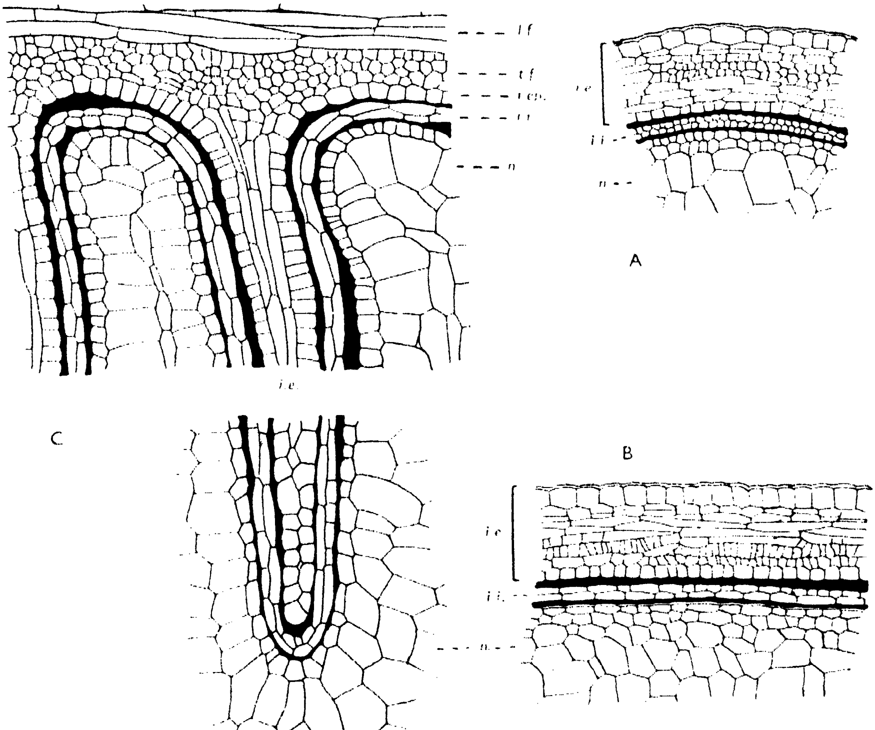

Fig. 10. *Annona squamosa.* Transverse (A) and longitudinal (B) sections of the developing wall of the young seed of Fig. 9, showing the beginning of the differentiation of the outer layer of longitudinal fibres and the inner layer of transverse fibres in the *i.e.*, and the separation of the hypodermis by periclinal divisions, × 225. C, a longitudinal section of the inner part of the *i.e.* of a half-sized seed, to show the formation of a rumination, × 225. *i.ep.*, inner epidermis of *i.e.*; *l.f.*, longitudinal fibres; *t.f.*, transverse fibres.

A. muricata. The seeds are slightly larger and more compressed than in *A. squamosa* and have acute edges. The aril is less prominent, not, or hardly, bulging beyond the general level of the testa, but it becomes yellowish ochre to brownish ochre through the darkening of the more thickened walls of the elongate hypodermal cells and of the interior cells.

Thus, the aril is even more rudimentary than in *A. squamosa.* Microscopically, the structure is essentially the same.

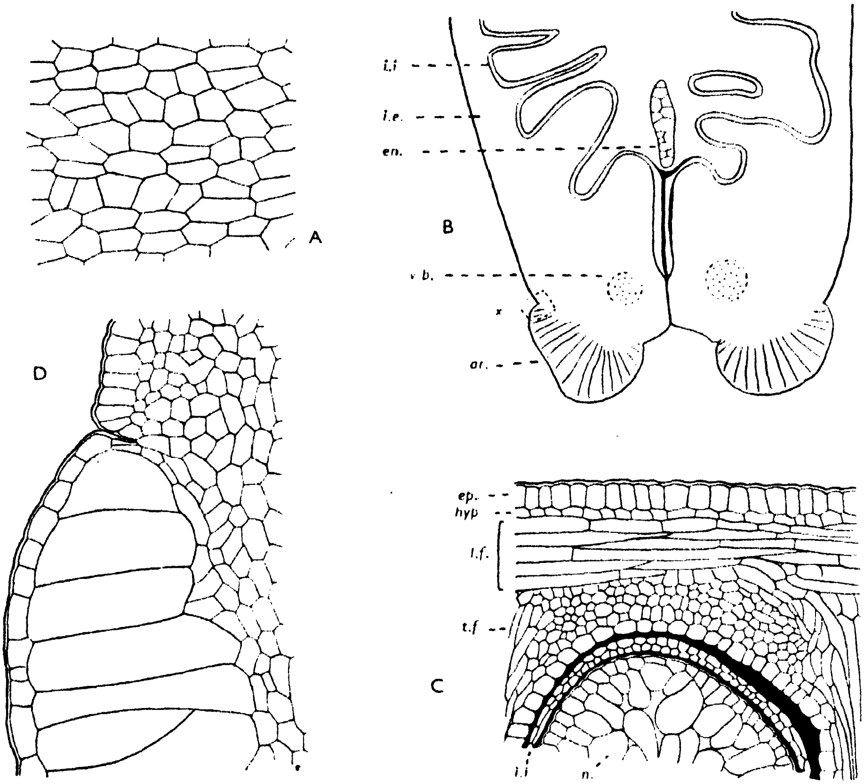

Fig. 11. *Annona squamosa.* A, the epidermis of the testa of the mature seed, in surface view, × 225; B, a transmedian longitudinal section of the micropyle of a half-grown seed, × *c.* 50; C, a part of the wall of the seed of B, × 225; D, the part *x* of the seed of B, at the junction of aril and testa, × 225. *ep.*, epidermis of *i.e.*; *hyp.*, hypodermis of *i.e.*; *l.f.*, longitudinal fibres of *i.e.*; *t.f.*, transverse fibres of *i.e.*

Desmos dasymaschala

The compound fruits of this very common, small Malayan tree are composed of a large number of stalked, moniliform, pulpy, indehiscent pods containing 1–5 seeds, and constricted between the seeds. The seed structure appears typical of that of many Annonaceae, as *Annona*, *Polyalthia* and *Xylopia* (in part).

Seeds. 10 × 6 mm., ellipsoid to subcylindric, subtruncate at the small hilum, shortly umbonate at the apex, covered over the sides by a thin, greyish black, finely mottled membrane, the raphe (surrounding the median plane of the seed) being pallid; *aril*: none; *hilum*: small, whitish, the *v.b.* of the raphe and the endostome slightly projecting (the exostome not contributing to the micropyle); *funicle*: none; *endosperm*: white, firm, with pale brown ruminations developed as thin transverse plates from the sides of the seed, giving the endosperm a cruciate form in t.s. of the seed.

Testa. Derived from *i.e.*, and composed of: (1) an epidermis of isodiametric, thin-walled cells, many of them (in groups) containing dark blue or indigo sap (anthocyan) and, thus, with the patches of colourless cells causing the mottling of the seed; (2) a hypodermis of small, thin-walled, colourless cells (easily overlooked), mostly with a short, oblong crystal; (3) a layer of longitudinal, or somewhat oblique fibres, thick-walled, much pitted, and colourless; (4) an inner layer of transverse fibres, less strongly thickened and pitted, also colourless: this inner layer entering into the ruminations.

Inner integument. Soon disintegrating, never more than 2 cells thick, without oil-cells.

Ruminations. Formed by the inner integument and, principally by the inner layer of transverse fibres of the outer integument.

Nucellus. Persisting in the mature seed only as the brown cuticular lamina and as oil-cells, derived from the hypodermis (as in *Annona*) and more or less embedded in the outer part of the endosperm. The oil-cells are very conspicuous in the fresh tissue, because of the orange colour of the oil.

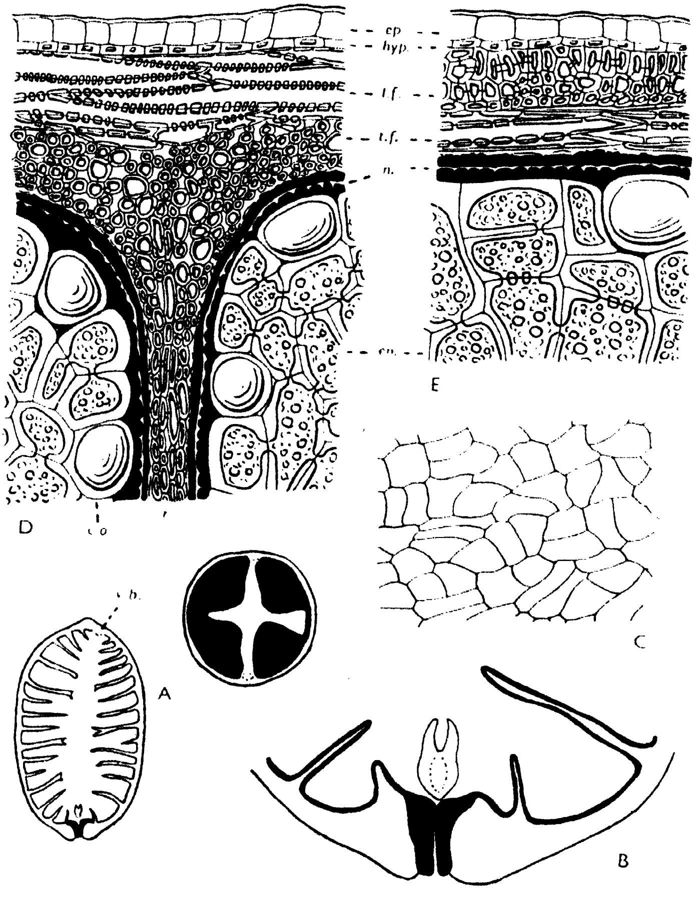

Fig. 12. *Desmos dasymaschala.* A, a transmedian longitudinal and a transverse section of the seed, the extent of the *i.i.* shown in black (as extending over the ruminations), × 1½; B, a transmedian longitudinal section of the micropyle of a mature seed, the endostome and *i.i.* in black, × 15; C, the epidermis of the testa, × 225; D, a longitudinal section, and E, a transverse section, of the wall of a mature seed, D, showing a rumination, × 225. *ep.*, epidermis of *i.e.*; *hyp.*, hypodermis of *i.e.*; *l.f.* and *t.f.*, longitudinal and transverse fibres of *i.e.*; *n.*, cuticle of nucellus.

Endosperm. Composed of cells with thickened, pitted walls and containing both many fine oil-drops and many rather small starch grains.

Subhilar tissue and the thickened exostome consist of a mass of sinuous fibres, as in *Canangium*.

The *i.e.* of the ovule is composed of four cell-layers, the *i.i.* of two cell-layers: thus, unlike *Annona*, there is no inner epidermis to the outer integument.

At maturity of the seed, which develops as in *Annona*, the outer two layers (epidermis, hypodermis) of the testa form a soft membrane which does not become pulpy as in *Annona*, but breaks down and dries up.

Desmos chinensis and Desmos dumosa

These are climbing plants with very differently shaped flowers from those of *D. dasymaschala*. In general, their seeds resemble those of *D. dasymaschala*, but with the following quantitative differences:

(1) The fibrous layers of the testa are much thinner, each being only 2–3 cells thick.

(2) The seeds are not mottled but evenly pale to medium brown, with firm, smooth surface, because the epidermis and hypodermis of the testa are so thin that they soon shrivel up, the pale brown, outer and slightly thickened walls of the epidermal cells forming the continuous pellicle over the seed.

(3) The endosperm cells have more oily contents: *D. chinensis* has very few and small starch grains in these cells, but they are conspicuous in *D. dumosa*.

The ovules have the same construction as in *D. dasymaschala*, but I observed in *D. chinensis* that the *i.e.* is not fully developed in the open flower but leaves the *i.i.* exposed over its distal part (Fig. 13 B).

The seed of *D. dumosa* closely resembles, both macroscopically and microscopically, that of *Xylopia caudata*.

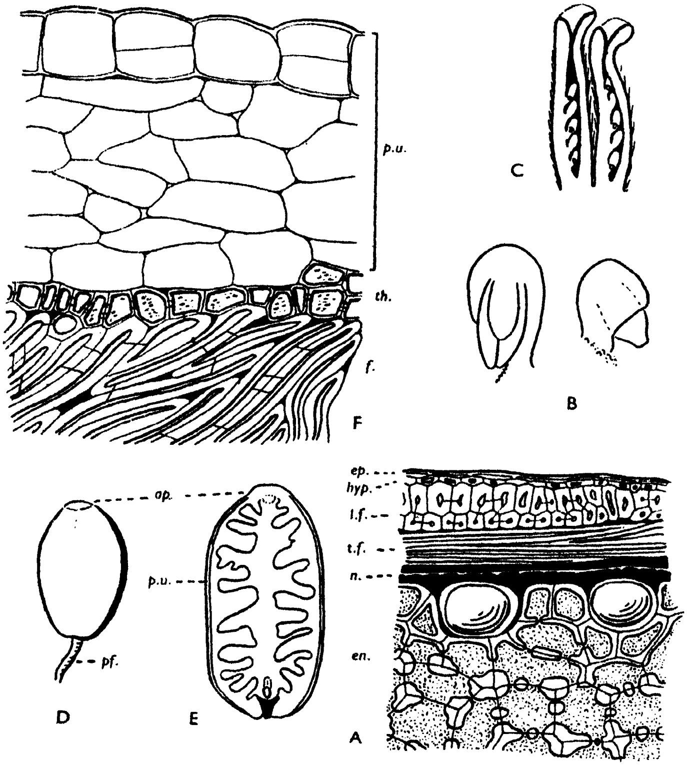

Fig. 13. *Desmos chinensis* (A–C) and *Xylopia fusca* (D–F). A, a transverse section of the testa of a mature seed, × 225; B, ovules of the mature flower (the section being oblique), × 40; C, longitudinal sections of two mature carpels, × 15. D, a seed, × 2; E, transmedian longitudinal section of a seed, the endostome in black, × 3; F, a transverse section of the outer part of the testa, × 225. *ap.*, the smooth brown apical patch; *ep.* and *hyp.*, epidermis and hypodermis of *i.e.*; *l.f.* and *t.f.*, longitudinal and transverse fibres of *i.e.*; *pf.*, the pseudo-funicle; *p.u.*, the pulpy outer layer of testa; *th.*, the row of short thick-walled cells (? hypodermis).

Xylopia fusca, Xylopia caudata and Xylopia malayana

The fruits of these species are a cluster of dorsally dehiscent, several-seeded follicles, opening along the abaxial suture. The endocarp is bright pink to blood-red, usually rather fleshy or flaky with the seeds at first more or less embedded in it: then, as the endocarp dries or breaks up, the seeds hang out on 'pseudo-funicles', consisting of their vascular strands which penetrate the thickened endocarp: actually, the seeds are sessile (as usual in the family). The three species show an interesting transition between the *Annona* type and the *Desmos* type, that of *X. fusca* probably resembling most closely in construction the arillate seeds of *Xylopia* spp. which occur in Tropical Africa and South America. None of these three species of Malayan trees has an aril.

The following is a description of the seeds of *X. fusca*:

Seeds. 11–12 × 6 mm., ellipsoid-subcylindric, covered with a greenish blue to greyish blue waxy-fleshy pellicle, which eventually dries and flakes off to expose the dull brown, rugose, woody inner testa: pseudo-funicles (placental *v.b.*) 4–6 mm. long, brown, hanging.

Testa. As in *Desmos* but: (1) with a thick parenchymatous outer layer, 6–8 cells thick, becoming pulpy: the epidermis slightly thick-walled; (2) without a hypodermis with crystal cells; (3) a thin, often single, layer of isodiametric, rather irregular cells with slightly thickened and pitted walls immediately internal to the pulpy layer: (possibly this layer is homologous with the crystal-bearing hypodermis of *Annona* and *Desmos*); (4) a very thick layer of variously directed and curved, long, thick-walled fibres with brown gummy contents; this layer also forming the thick ruminations; (5) with a smooth, woody, brownish patch at the opposite end of the seed from the micropyle, composed of the woody layer of the testa and only 1–2 rows of thin-walled parenchymatous cells on the outside (that is, this apical patch has the structure of the testa of *Desmos chinensis* and *D. dumosa*).

Endosperm. Oily, the cells thick-walled, without starch.

In *Xylopia caudata* and *X. malayana*, the seeds are smaller (6–7 mm. long) and have no apicular (anti-micropylar) or brown patch because the whole seed has the structure of the apical patch of *X. fusca* or, rather, of *Desmos chinensis*. The outer, thinly pulpy, layer of the testa consists of an epidermis with blue sap in the cells, and a hypodermis of crystal-bearing cells: and the fibrous layer is double, also as in *D. chinensis*. The inner woody shell of the testa is smooth, thin and pale brown as in *Desmos*, and the ruminations are also thin. In *Xylopia caudata* the endosperm is oily, without starch, but in *X. malayana* it is starchy: the cells are thick-walled in both species.

Thus, it appears that the seeds of *Desmos*, as of *Xylopia caudata* and *X. malayana*, can be considered as the seeds of *X. fusca* with the apical patch extended over the whole seed.

Polyalthia purpurea

The compound fruits of this genus consist of a cluster of stalked 1-seeded berries or drupes with leathery or fleshy wall and a single large seed (rarely more than one). The testa of the seed is almost wholly fibrous, even the cells of the epidermis having this form, so that the seeds have the character of overgrown bodies, without a limiting epidermal palisade, as I will explain in a later paper. They resemble, in fact, overgrown seeds of *Desmos*.

Seeds. 1·6–1·8 × 1·3–1·5 cm., ellipsoid, light brown, with darker depressed raphe, finely transversely fibrillose (the testa being without longitudinal fibres); *hilum*: small, yellow; *endosperm*: white, hard, starchy, traversed by thin, pale brown ruminations as plates from the sides of the seed; *aril*: none.

Testa. Thin, little differentiated, composed entirely of long transverse fibres with thick, pale brownish walls and scattered pits, the epidermal cells also elongate and fibriform (though some short and undifferentiated).

Inner integument. Reduced to a single layer of isodiametric or slightly transversely elongate cells with slightly thickened, pale brown walls: some of the cells, especially in the ruminations, becoming oil-cells.

Ruminations. Formed by the *i.i.*, but the outer integument extending into the bases of the larger plates.

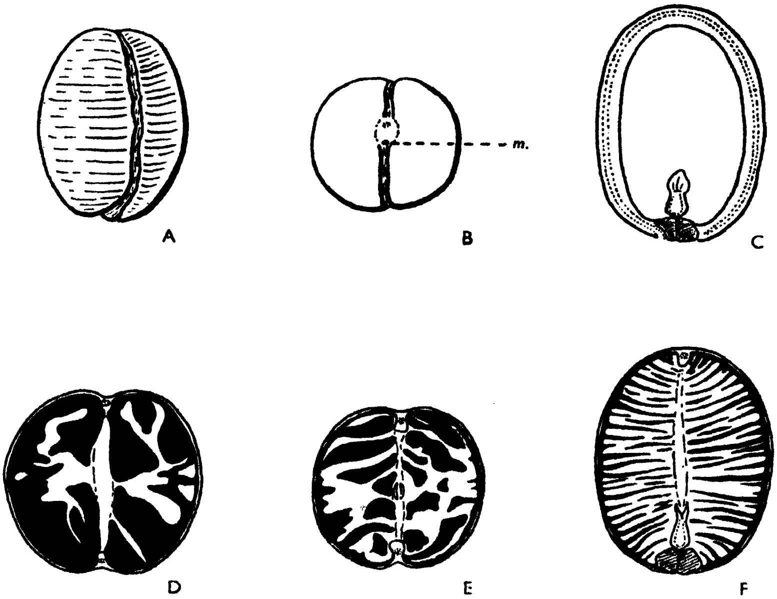

Fig. 14. *Polyalthia purpurea.* A, seed showing the raphe, × 1½; B, hilum of the seed, × 1½; C, median longitudinal section of the seed, showing the *v.b.* (dotted) and the subhilar tissue (hatched), × 2; D, E, transverse sections of the seed, showing the ruminations (in black), × 2; F, transmedian longitudinal section of the seed showing the ruminations (black), the subhilar tissue (hatched), and the clear central plate of endosperm, × 2.

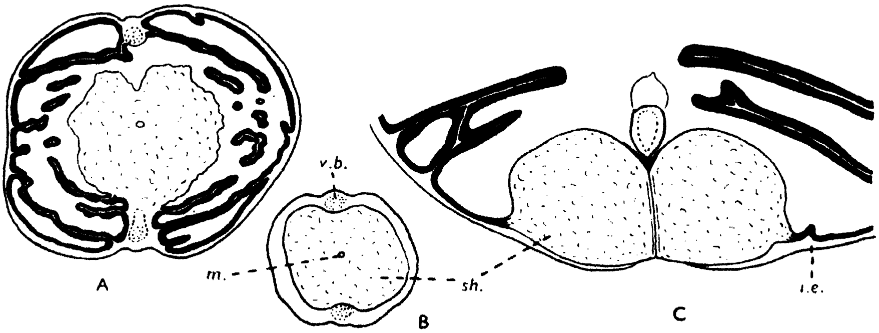

Fig. 15. *Polyalthia purpurea.* A, B, transverse sections of the seed across the subhilar tissue, B, near the micropyle, × 7; C, a transmedian longitudinal section of the hilar end of the seed, × 15. (The ruminations shown in black.) *sh.*, the subhilar tissue of the *i.i.*

Nucellus. Represented only by the brownish cuticle, or absent altogether from the mature seed.

Endosperm. Composed of thick-walled cells, full of small starch grains, deeply pitted; the outer 1–2 layers of cells, next to the testa, thin-walled and not pitted, but also full of starch.

Subhilar tissue. Consisting of a sticky, ochre-yellow mass of substellate cells (with very short arms), full of oily-smeary protoplasm, the mass derived entirely from the thickened endostome tissue and separating from the other tissues at maturity.

Structure of the ovule. The ovule is constructed as in *Anaxagorea*. The *i.e.* is 4–5, or 6 cells thick, the *i.i.* only 2 cells thick, but the *i.i.* is longer than the *i.e.*, so that the endostome projects. The *v.b.* of the ovule is embryonic in the open flower.

Development of the seed. Essentially as in *Anaxagorea* and *Annona*, but there is no trace of an aril and the outer integument does not differentiate into distinct layers: it has no crystal cells or pulpy tissue. A small group of dark brown cells appears at the chalazal end of the young seed, but does not persist (as happens in the Magnoliaceae).

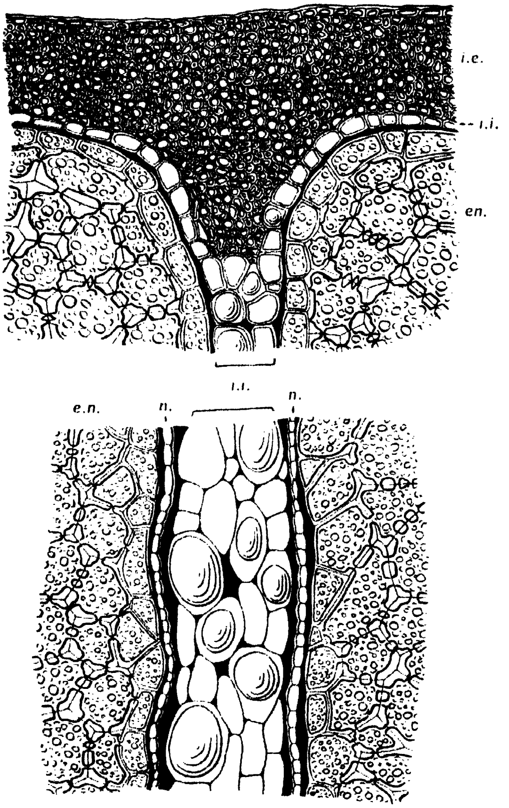

Fig. 16. *Polyalthia purpurea*. A part of a longitudinal section of the testa, showing a rumination, × 225.

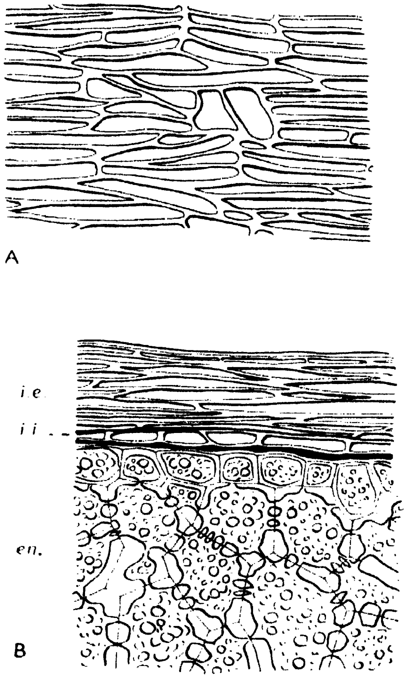

Fig. 17. *Polyalthia purpurea*. A, the epidermis of the testa in surface view, × 400; B, a transverse section of the testa, × 400.

Vascular supply of the seed. A single vascular bundle passes round the raphe of the seed and ends blindly before the micropyle without a plexus or branching.

(*Note.* The material for this study came from the tree near the Sundial Terrace in the Singapore Botanical Garden.)

Artabotrys odorata

The seed of this species has two integuments, but the structure of the outer one resembles that of the middle integument of *Xylopia Curtisii* and *Mezzettia leptopoda* (described later): that is, there is a large-celled epidermis, a small-celled hypodermis below which is a layer of fibres perpendicular to the surface of the seed, and internally a layer of oblique fibres. The ruminations are compound, consisting of both integuments. There is no aril.

(I think the seeds need much closer examination, especially in other species.)

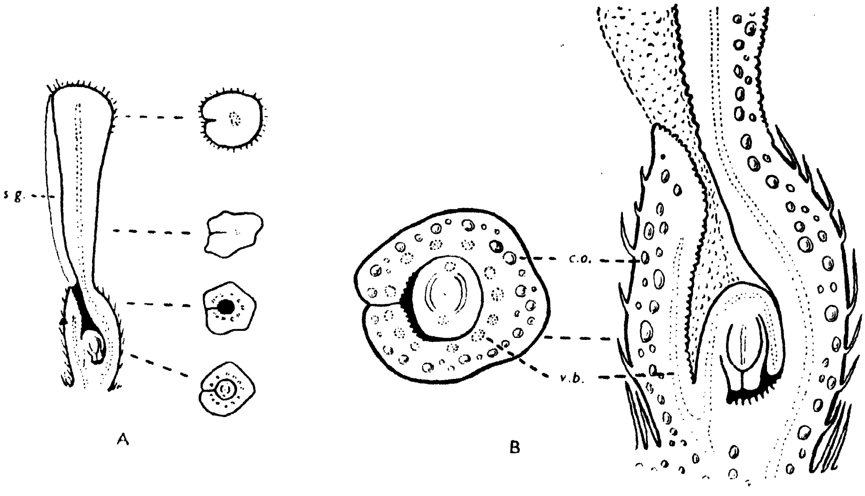

Fig. 18. *Polyalthia purpurea*. A, longitudinal and transverse sections of the carpel of the mature flower, × 15; B, the base of the carpel, showing the open cavity of the carpel and its papillate, inner ventral surface, × 50. (*v.b.* dotted.) *s.g.*, the stylar groove.

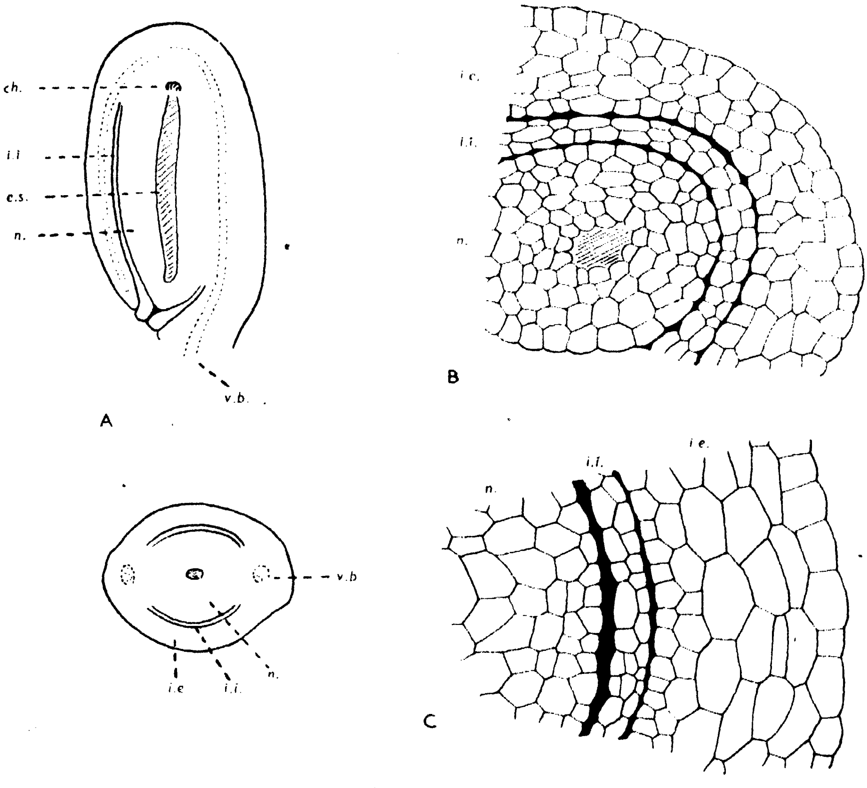

Fig. 19. *Polyalthia purpurea*. A, longitudinal (oblique or tangential) and transverse sections of a very young seed, × 50; B, transverse section of the ovule of the mature flower, × 400; C, transverse section of the young seed shown in A, × 400. *ch.*, the group of brown chalazal cells; *e.s.*, the embryo sac.

Popowia

Popowia nervosa has seeds constructed exactly as those of *Polyalthia purpurea*. The endosperm has starch and there are oil-cells in the ruminations of the *i.i.*

On the other hand, the seed of *Popowia tomentosa* (with 1–2 seeds per carpel) has a testa composed of several layers of parenchyma outside layers of longitudinal and oblique fibres, evidently like the seed of *Xylopia fusca*.

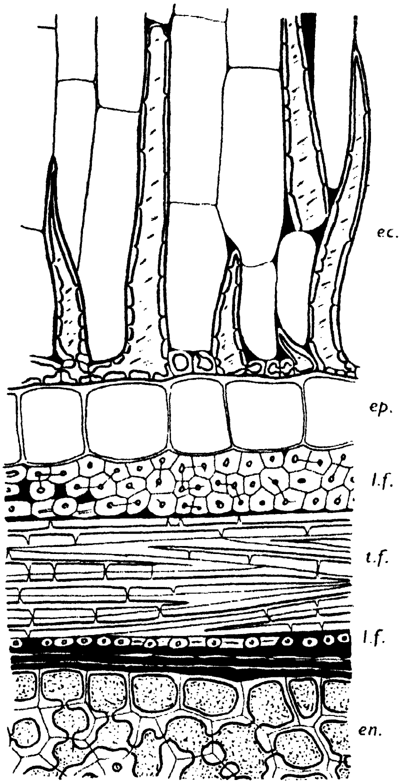

Fig. 20. *Alphonsea elliptica.* A transverse section of the testa and adjacent part of the endocarp of a full-grown seed, × 225. *ec.*, endocarp, with pulpy and fibrous cells; *ep.*, epidermis of *i.e.*; *l.f.* and *t.f.*, longitudinal and transverse fibres.

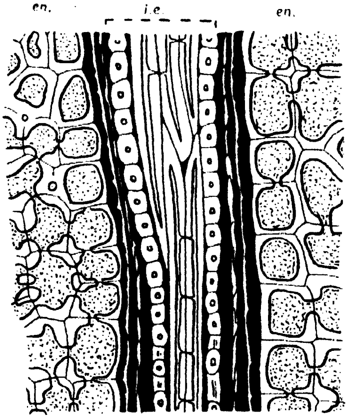

Fig. 21. *Alphonsea elliptica.* Transverse section of a rumination of a mature seed, showing the two inner fibrous layers of the *i.e.* composing the rumination, × 225.

Alphonsea elliptica

The fruits of this village tree of Trengganu are clusters of short, banana-like, fleshy indehiscent pods with pulpy mesocarp and several (3–10) large, brown, oblong-flattened seeds inseparable from the fibrous pulpy endocarp. But the seeds have a well-defined testa, as revealed by microscopic study. The cells of the endocarp are arranged in rows perpendicular to the surface of the seed: most of the cells are oblong, pulpy and thin-walled, but the cells of the epidermis of the endocarp, pressed tightly against the uneven testa of the seed, are thick-walled and drawn out into thick-walled hairs which project for distances of 5–1 mm. into the pulpy endocarp, thus binding it to the testa. The seeds evidently have a long hilum (as in *Xylopia Curtisii*, see later), but I was unable to make out its exact form in the alcohol material which I had. There is no aril.

Testa. Composed of: (*a*) a large-celled epidermis, the cells with brown contents, collapsing at maturity of the seed; (*b*) a layer of longitudinal fibres, 2–3 cells thick; (*c*) a thicker layer of transverse or oblique fibres; (*d*) an inner layer of longitudinal fibres, only one cell thick.

Ruminations. Composed of both *i.e.* and *i.i.*, the two inner-fibrous layers of the *i.e.* entering the ruminations.

Endosperm. Very thick-walled, with oily contents (no starch). There appear to be no oil-cells in the integuments or nucellus.

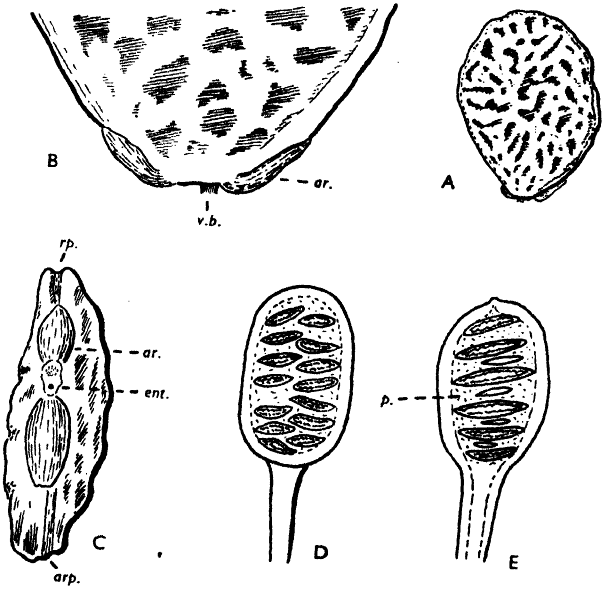

Fig. 22. *Canangium odoratum.* A, seed, ×3; B, base of the seed, ×10; C, hilum of seed, ×7; D, E, tangential and median longitudinal sections of the fruiting carpel, ×1. *ar.*, aril; *arp.*, antiraphe; *ent.*, endostome; *p.*, endocarp pith of fruit; *rp.*, raphe.

Canangium odoratum

The seeds of this very common and much-cultivated tree of tropical Asia show a remarkable feature, which I have discovered also in the following two species, *Xylopia Curtisii* and *Mezzettia leptopoda*. The testa is formed, practically entirely, from a new tissue which I call the *middle integument*, arising after fertilization and developing on the sides of the young seed between the outer and inner integuments. The ovule, and the young seed, are perichalazal, as in all the foregoing examples: that is, the chalaza extends all round the seed except for a short part near the micropyle where the two integuments are completely free, the outer sheathing the inner. Thus the middle integument develops only on the sides of the seed, in the gap between the two integuments, and as a sort of mattress between the two: its growth, however, is basipetal, as general in these Annonaceous seeds. The middle integument has the fibrous structure of the outer integument of the foregoing species, and it forms, with the inner integument, the ruminations. It usurps, completely, the functions of the outer integument.

The fruits of *Canangium odoratum* resemble those of *Polyalthia*, being clusters of drupe-like indehiscent follicles, but they contain many, transversely arranged, seeds (up to 14).

Seeds. 9–11 × 6–7 × 2–2·5 mm., flattened, with ovate outline, light brown, covered with a whitish membrane, the inner brown tissue hard and shallowly pitted on the surface; *hilum*: very small; *aril*: small, distinct, as a short, yellow or brownish white horny tongue-like lobe reflexed on each side of the hilum or each edge of the seed; *endosperm*: white, with short, brown ruminations.

Testa. Consisting of the following discrete layers: (1) an outer white membrane, representing the *i.e.*; (2) a thick, brown, woody layer, representing the *i.m.* (*integumentum medium*); (3) an extremely thin, inner membrane against the endosperm and representing the *i.i.*

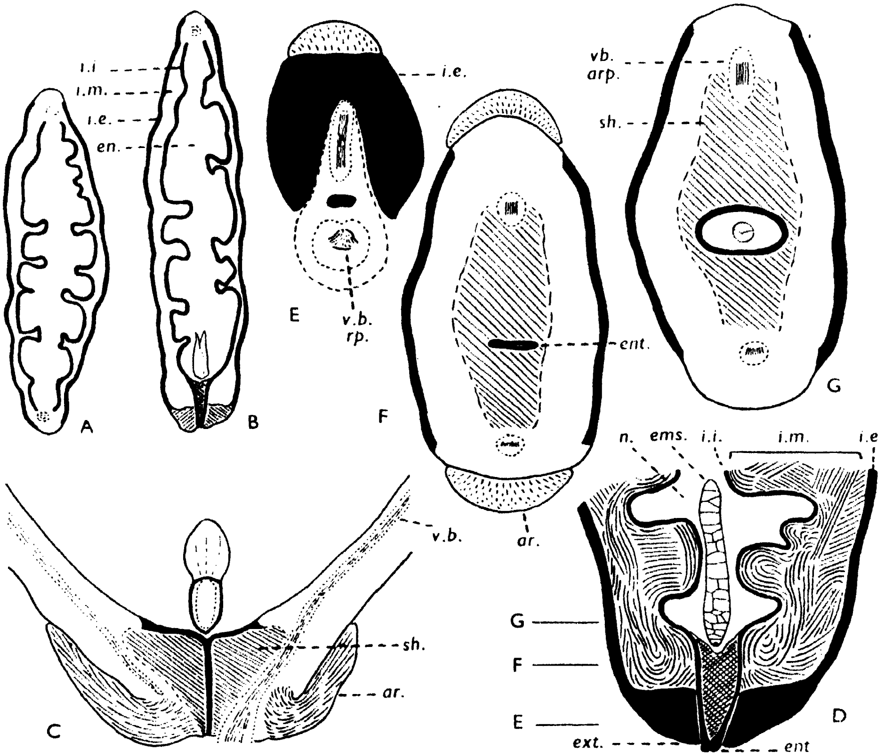

Fig. 23. *Canangium odoratum.* A, B, transverse and transmedian longitudinal sections of full-grown seeds (the thickness of *i.i.* exaggerated), × 7; C, median longitudinal section of the hilum and micropyle, × 15; D, transmedian longitudinal section of the micropyle of an immature, nearly full-sized seed, × 25; E, F, G, successive transverse sections at the levels shown in D, × 25. *ar.*, aril; *arp.*, antiraphe; *ems.*, embryo sac with incipient endosperm and minute embryo at the base; *ent.*, endostome; *ext.*, exostome; *rp.*, raphe; *sh.*, subhilar tissue; *i.i.*, inner integument; *i.m.*, middle integument; *i.e.*, outer integument.

(1) The outer integument consists of some four rows of cells: (*a*) an outer epidermis of shortly elongate cells, forming an irregular pattern in surface view; (*b*) a single row of transverse or oblique fibres, crossing those of the preceding layer; (*c*) a single row of longitudinal or oblique fibres; (*d*) an inner epidermis of thin-walled, parenchymatous cells, collapsing as the seed dries.

The outer integument is attached to the raphe, or chalaza, all round the seed, but is free on the sides of the seed. At the exostome, it is thickened, by periclinal division of the two middle cell-layers, giving a complex of fibres round the hilum.

(2) The middle integument consists of four layers: (*a*) a thin-walled epidermis, many of the cells with short crystals; (*b*) a layer, 2–4 cells thick, of longitudinal fibres with brown walls; (*c*) a

thicker layer of alternating bands of longitudinal, or oblique, and transverse fibres, the outer ones with pale brown walls, those of the inner ones colourless; (*d*) an inner epidermis of small, isodiametric, slightly thick-walled cells.

(3) The inner integument is only 2 cells thick, as in the ovule, and soon collapses. It has no oil-cells.

Hilum: with the broken end of the *v.b.* of the raphe shortly projecting, and the fine orifice of the endostome visible at the apex of the light brown woody cone which forms the subhilar tissue. The endostome is flattened strongly in the transmedian plane of the seed (at right angles to the plane of flattening of the seed itself): its wall is 2 cells thick, except at the end where it is 3–4 cells thick. The periphery of the hilum is a white horny tissue, composed of variously interwoven fibres derived from the tissue of the raphe and antiraphe and of the adjoining outer integument. The middle integument does not reach the surface to form a 'mesostome', but it forms very largely the woody cone of the subhilar tissue. This is, in the growing seed, a mass of thin-walled aerenchyma (cells isodiametric, but not stellate), connecting with the aerenchyma of the raphe and thus with that of the fruit-wall. On maturing of the seed, the cells become very thick-walled, woody and, on drying up, contract into the light brown woody cone which forms the plug closing the hilum.

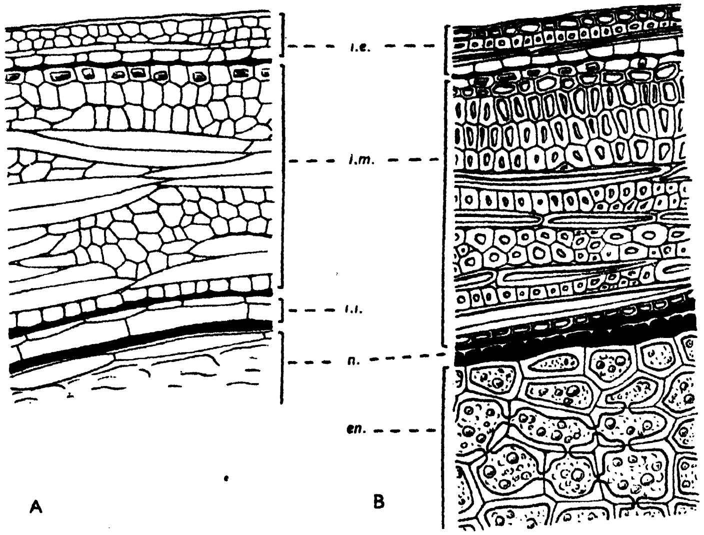

Fig. 24. *Canangium odoratum.* A, transverse section of the testa of an immature, but full-grown, seed (the fibres still thin-walled), × 225; B, the same from a mature seed, × 225.

Aril. Consisting of two small discrete lobes, like a pair of moustaches, from the hilum, composed of large elongated cells with slightly thickened walls and oily, often yellowish, contents; the surface being covered with an epidermis of low, shortly elongate cells (absent from the tips of the lobes).

At the base of the aril lobes, the cells are narrow and elongate and, on ripening of the seed, they become very thick-walled so that the aril lobes are not detachable. These cells connect with the mass of criss-cross fibres surrounding the hilum.

Ruminations. Composed of massive truncate foldings of the middle integument, covered by the *i.i.*, the fibres of the *i.m.* taking complicated curved directions.

Nucellus. Only the brownish cuticle persisting in the mature seed.

Endosperm. Composed of thick-walled pitted cells with oily contents (no starch).

Embryo. Composed of thin-walled cells.

Structure of the ovule. The ovule is similar to that of the preceding genera (and, as in *Polyalthia*, the apex of the carpel is often open at the time of pollination, the pollen tubes growing down the sides of the stylar groove, not in the stylar tissue: indeed, the ovary suture may be open nearly to the base). The *i.e.* consists of four layers of cells, the *i.i.* of two cells, and at the opposite end of the ovule from the micropyle there is a thick mass of tissue on either side, between the two integuments, as the rudiment of the *i.m.* These lobes of the *i.m.* develop long after the other two integuments in the young flower. The *v.b.* of the antiraphe is scarcely differentiated until fertilization of the ovule.

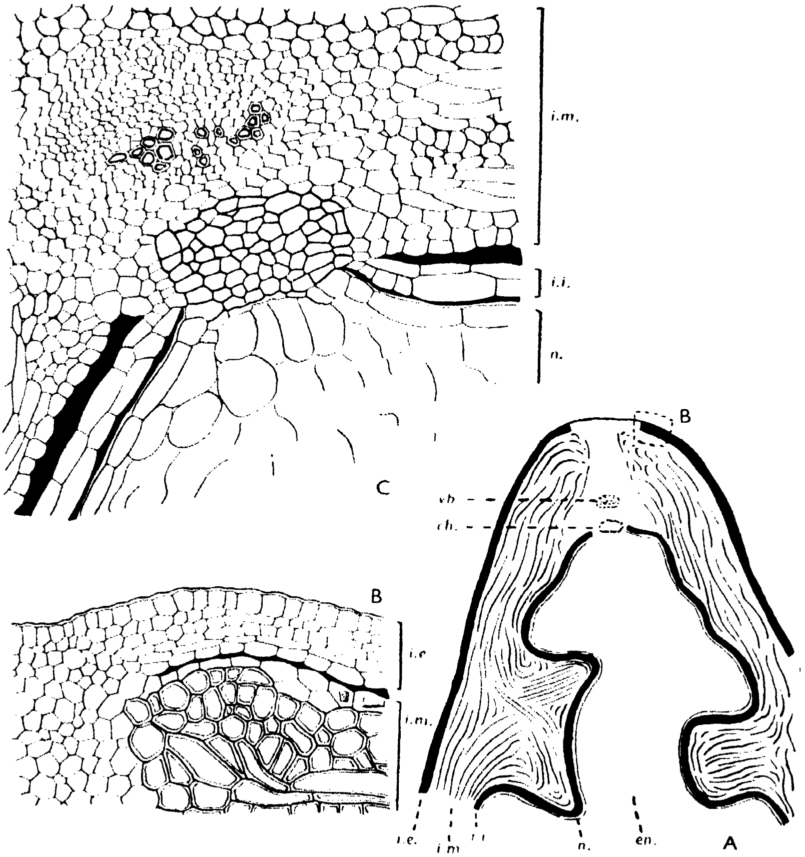

Fig. 25. *Canangium odoratum.* A, transmedian longitudinal section of the opposite end of the seed from the micropyle (the seed full-grown, but immature), the *v.b.* surrounded by a parenchymatous sheath, × 25; B, the part marked B in A, × 225; C, the part of A containing the *v.b.* and the group of chalazal cells (*ch.*) with slightly thickened walls, × 225.

Development of the seed. On fertilization, the ovule grows mainly by interstitial growth from the end opposite to the micropyle (in the region marked *aa–bb* in Fig. 29A): thus, the seed develops basipetally and, as the micropyle is thrust farther from the opposite end, so the rudimentary humps of the *i.m.* develop basipetally into plates of tissue on the sides of the young seed. Thus it is that the chalaza, where the integuments are attached to the raphe, extends all round the seed except near the hilum where the three integuments are completely free.

The nucellus enlarges greatly, as described in the preceding genera. The endosperm develops into a cigar-like body along the centre of the seed, following the form of the embryo sac, before extending to the sides between the ruminations. The hypodermal cells of the nucellus enlarge considerably just before they collapse and give way to the endosperm. There are no oil-cells in the nucellus.

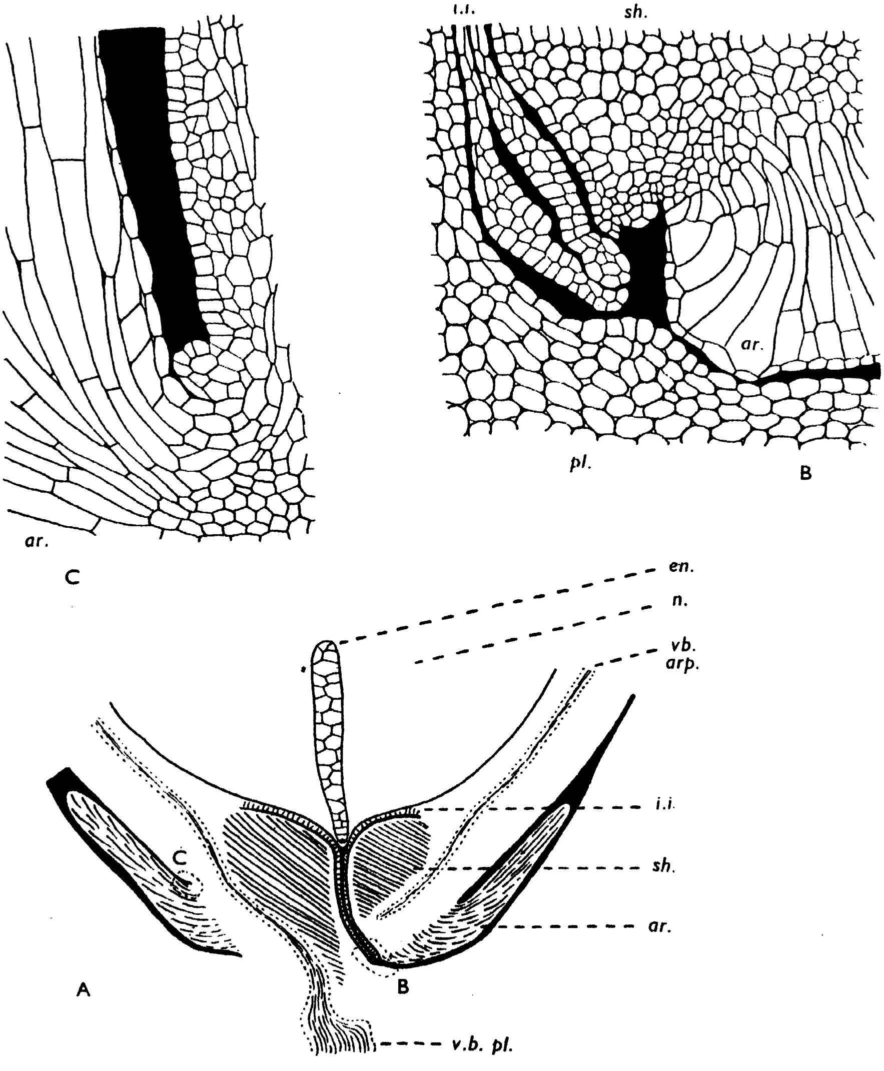

Fig. 26. *Canangium odoratum.* A, median longitudinal section of the hilum and micropyle of a seed two-thirds grown, × 25; B, C, magnifications of the parts marked in A, × 225. *ar.*, aril; *arp.*, antiraphe; *en.*, incipient endosperm; *pl.*, placental tissue; *sh.*, subhilar tissue.

The aril lobes arise at an early stage, long before the seed is full-size.

The cells of the outer integument do not undergo periclinal divisions, so that the *i.e.* of the testa is composed of the same four layers: the cells divide anticlinally, enlarge, and elongate, thus keeping pace with the growth of the seed.

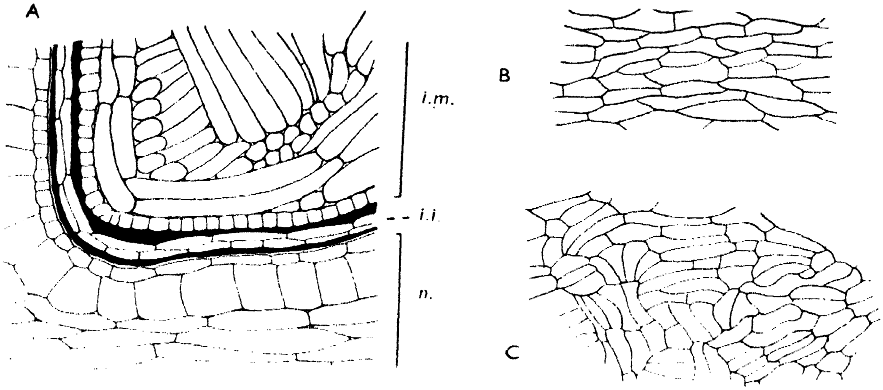

Fig. 27

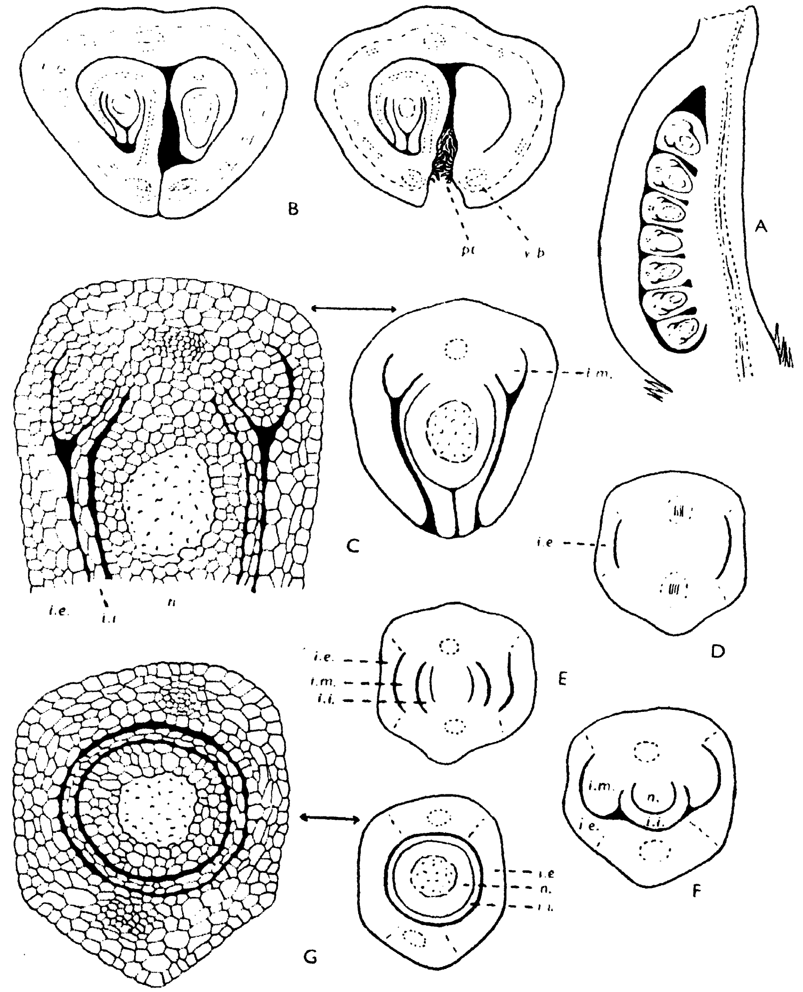

Fig. 28

Vascular supply of the seed. There is a single, unbranched, vascular bundle in the raphe-antiraphe, ending blindly before the micropyle: it does not send any branches to the aril lobes.

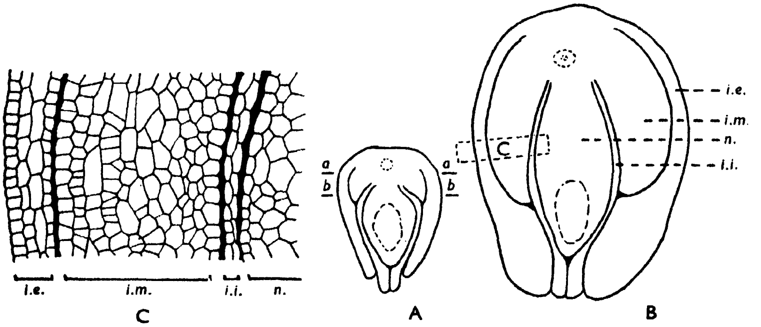

Fig. 29

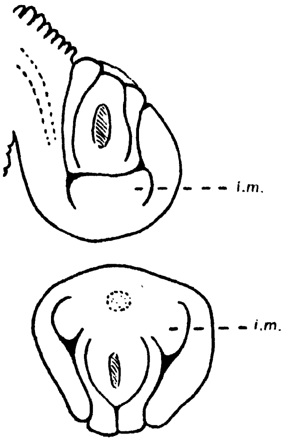

Fig. 30

Fig. 29. *Canangium odoratum.* A, transmedian longitudinal section of the ovule of an open flower, *aa–bb* the region where the ovule grows basipetally to form the bulk of the seed, × 40; B, similar section of fertilized ovule developing into the seed, × 40; C, the part marked C in B, × 225.

Fig. 30. *Mezzettia leptopoda.* Tangential longitudinal and transmedian longitudinal sections of the ovule of the mature flower, × 40.

Mezzettia leptopoda

I have not been able to follow the development of the seed in this genus, but the ovule of the mature flower shows the incipient middle integument just as in *Canangium* (Fig. 30). The large seed, 3·5 × 2·8 × 1·8 cm. (2 per drupe), closely resembles that of the following species *Xylopia Curtisii*, having the same shape, linear hilum with woody plug, and hard, thick, woody walls. These, as in *X. Curtisii*, are clearly composed mainly of the middle integument, but they are thicker and have a distinctly columnar-like layer of fibres at the outside immediately below the epidermis of the *i.m.* The middle layer of *i.m.* consists of interlocking bundles of fibres running in various directions. The endosperm is oily, without starch: the ruminations are stout, fibrous and formed mainly by the *i.m.*

Xylopia Curtisii

This species belongs to a section of *Xylopia* with large drupe-like, pulpy, indehiscent carpels (one per flower), each with many flattened seeds with oblong hilum. I have examined the fresh flowers, and immature seeds from dried material and find that there

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Legends to Figs. 27 and 28

Fig. 27. *Canangium odoratum.* A, the apex of a rumination in a nearly full-grown, but immature seed, the nucellus (*n.*) still present, and the fibres of the *i.m.* unthickened, × 225; B, the epidermis of the aril, in surface view, × 225; C, the epidermis of the outer integument, in surface view, × 225.

Fig. 28. *Canangium odoratum.* A, median longitudinal section of a carpel just after fertilization, showing the incipient middle integument in the ovules (in transmedian longitudinal section), × 25; B, transverse sections of the mature flower, *pt.*, the pollen tubes germinating in the open suture of the right-hand section, × 50; C, transmedian longitudinal section of the ovule immediately after fertilization, × 75 (the magnification on the left, × 225); D–G, successive transverse sections of such an ovule as shown in C, taken from the chalaza toward the micropyle, showing how the integuments are free only near the micropyle, × 75 (the left-hand magnification of G, × 225).

is a middle integument composing most of the testa, as in *Canangium* and *Mezzettia* (Fig. 30 would serve also for *Xylopia Curtisii*). So different are these features from *X. fusca*, *X. caudata*, *X. malayana* and other species of the same alliance, that one may doubt if *X. Curtisii* is correctly placed in the same genus, or even in the same close circle of affinity.

The structure of the testa resembles that of *Canangium* in general, but the middle integument is much thicker and woodier and forms stouter, fibrous ruminations: the outer epidermal cells of *i.m.* are full of broken rod-like crystals. The hilum and exostome, however, are narrow and linear, or flattened; and a large flattened woody plug, derived from the *i.i.*, surrounds the endostome. It is possible that the *v.b.* of the antiraphe enters the placenta as in *Anaxagorea*. There is no aril. There appear to be no oil-cells in the nucellus, or *i.i.* Whether the endosperm is oily or starchy, I do not know because I have not studied ripe seeds.

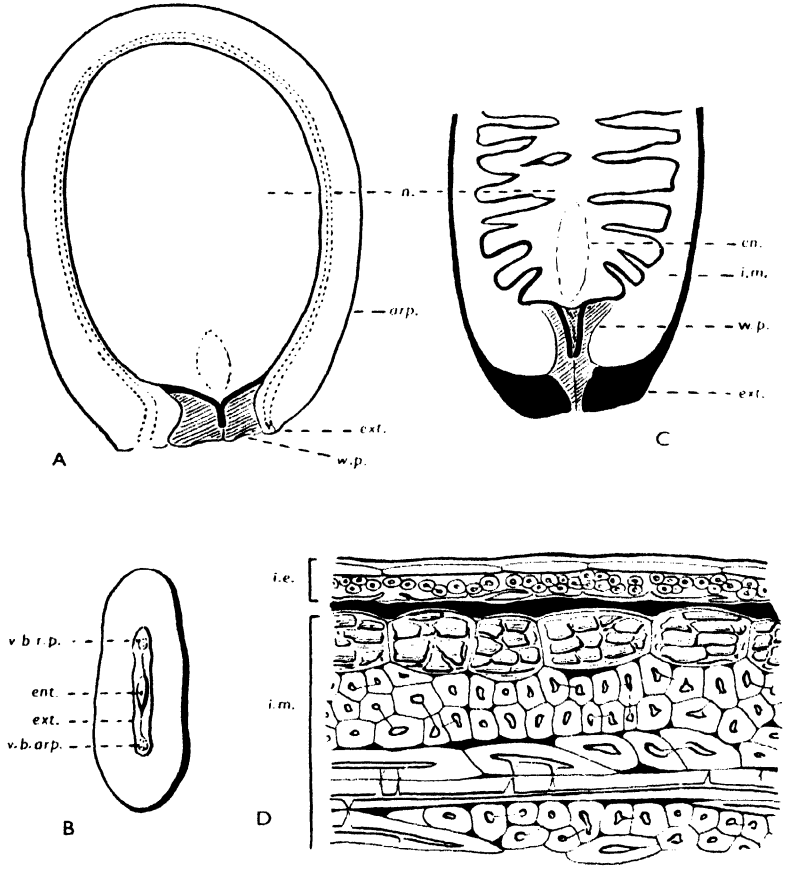

Fig. 31. *Xylopia Curtisii*. A, B, a median longitudinal section and hilar view of a full-sized, immature seed, × 2; C, transmedian longitudinal section of the hilum of B, × 4; D, transverse section of the outer part of the testa of a mature seed, × 225. *arp.*, antiraphe; *ent.*, endostome; *ext.*, exostome (as limit of hilum); *rp.*, raphe; *w.p.*, woody plug round endostome.

MORPHOLOGICAL PRINCIPLES

Postponed development

The nucellus is generally considered homologous with the prothallus of Pteridophyta. The great development of the nucellus in the Annonaceous seed shows that the production of the prothallus has been postponed until after fertilization. The morphogenetic factor of the prothallus has not been lost, but the time at which it becomes effective in the life cycle has been delayed. Alternatively, it may be held that the gametophyte is precocious and that the times of maturity and fertilization have been advanced. The relative displacement, one way or another, causes the precocity and, therefore, the embryonic appearance of the ovule in the flower at the time of pollination. After pollination the gametophyte develops and the new sporophyte takes advantage, as it were, of this delay to become a dormant embryo within the enveloping tissues of both the parent sporophyte and parent gametophyte. The process suggests that, originally, the seed was a prefertilization organ, as an elaborate sporangium the archaic construction of which can be read in the massive outer integument, or testa, of the seed. That is to say, the archaic sporangium had a thin inner integument surrounded by a massive, photosynthetic outer integument (cf. the stomata on the testa of the seeds of *Magnolia*, *Michelia*, *Myristica*) which, on maturity, developed woody, indehiscent and resistant walls, and was detached as such to be fertilized on the ground. Precocity and postponed development would lead to fertilization *in situ* in the second degree (gametophyte on sporophyte), and the modification of the sporangium into the modern seed. The process, indeed, appears to have reached its culmination in some orchids in which even the development of the ovule, beyond initiation, follows only after pollination.

The principle suggests, further, that the aril, developed in modern flowering plants only after fertilization, must have been a third and outer sporangial integument; and the picture, so conjured, is that of an animal-distributed megasporangium with pulpy outer envelope and woody middle layer, the micropyle being the canal for the antherozoids.

Transference of function

At a certain stage in the development of a multicellular organism, a cell or tissue differentiates. Before this happens, there must have been a critical cell division or cell divisions, leading to the distinction of the cells or tissues. If this division takes place at an earlier stage in development, then a greater bulk of tissue will become affected with the characteristic differentiation: if postponed, then a smaller amount of tissue (other things being equal). Alternatively, if the site of the critical cell division in the embryonic mass of cells is displaced externally or internally, even by a single cell-layer, the characteristic differentiation may affect a different organ.

In the case of the ovary, where many organs (such as ovary wall, placenta and ovule) differentiate in very close proximity or even contiguously in the minute flower-bud, such a displacement by a single cell-layer may result in a tissue characteristic differentiating in the placenta rather than the ovule, in the outer integument rather than the aril, or in the inner integument rather than the outer. This effect is shown well in the complicated seed structure of the Annonaceae with sharp distinctions in manner of development of the cell-layers of the ovule.

Take the instance of the oil-cells (*c.o.*, in the Figures). They occur in the middle cell-layer of the inner integument in *Anaxagorea javanica* (Figs. 3, 5); in the outer cell-layer of the *i.i.* in *Polyalthia purpurea* (Fig. 16); and in the hypodermis of the nucellus in *Annona* and *Desmos* (Figs. 7, 12 and 13). It will require only a very slight displacement, temporal or spatial, in the localization of this factor in the embryonic ovule, even by a single cell-layer, to cause the appearance of the oil-cells in the inner integument rather than the nucellus.

Secondly, the characteristic crystal-bearing layer of cells in the outer integument shows a similar deviation. They occur, typically, in the hypodermis of the *i.e.* in *Annona*, *Desmos* (Figs. 7, 12 and 13) and in *Xylopia caudata* and *X. malayana*. In *Canangium odoratum* and *Xylopia Curtisii* (Figs. 24, 31), however, they occur in the outer epidermis of the middle integument. One must assume that there has been a slight displacement in time or place of origin of this factor in the developing ovule which results in the character appearing in adjacent organs of the seed in these different genera.

Application of the same principle will explain the case of the pulpy or subgelatinous outer layer of the testa in *Annona*, *Desmos* and *Xylopia fusca*. It seems that the pulpy character of the aril, or outermost and third integument, has been transferred to the epidermis (and hypodermis, in some cases) of the usual outer or second integument. Thus, in Figs. 8 and 11, it can be seen how the aril of *Annona squamosa* is limited to comparatively few, greatly enlarged hypodermal cells, in contrast with the aril of *Canangium*. In the case of *Xylopia* there are many species with a large red aril more or less covering the seed. In *X. fusca* (Fig. 13) the aril is absent but the pulpy character of the aril is present in the epidermis and hypodermis of the outer integument. The distinction seems to be a matter of a single cell-layer in time or place of differentiation of the character. The apical patch of *X. fusca* (Fig. 13) indicates the part of the embryonic ovule in which this character does not differentiate: so that the seeds of *X. caudata* and *X. malayana* (as also those of *Desmos* and *Annona*) appear as basipetally developed homologues of the apical part of the seed of *Xylopia fusca*. The morphological differences indicate profoundly different modes of construction of their seeds which cannot be discovered without such visual or structural criteria to reveal them.

Finally, the middle or fourth integument of *Canangium*, *Mezzettia* and *Xylopia Curtisii* appears as a new growth, on the sides of the ovule, finalizing both the basipetal character of the growth of the Annonaceous seed and the displacement of the characters of the outer integument, so far as concern the crystal cells and the fibres, to this new embryonic mass. Indeed, the very basipetal, or antimicropylar, displacement of the seat of differentiation of the testa characters to the region of junction (or divergence) of the outer and inner integuments seems also to imply a combination of the factors evoking these testa characters with that which evokes annular or investing integumentary growth, so as to produce the new feature of a middle integument.

This principle of transference of function from one group of embryonic cells to another, as a deviation from the normal sequence of differentiation, has obviously very wide application both in botany and zoology. The problem of mesodermal, ectodermal and endodermal origin of organs has long exercised the ingenuity of academically morphological zoologists, as indeed that of membrane and investing bones in the skull. So, also, in botany, where embryology seems less important because of the lesser differentiation of tissues, the phyllomorphy of phyllodes and cladodes, as well as the petaloidy of corolla tubes, stamens and sepals, or the seed-like nature of the endocarp of drupes, are examples of the transference of function from one, normal, place in the meristem to an adjacent.

It is possible, however, that in some cases, at least, petals may be basipetal enations of secondary origin from the receptacle, just as the middle integument of these Annonaceous seeds. Once the universality of this principle is grasped, it is indeed difficult to see what else is needed to explain the variations on a common theme which characterize the adaptive radiation of plants and animals. However, other processes, such as physiological maturity and differences in relative growth rates cause apparently greater, if less profound, alterations: and to these must be added the still more effective principle of postponed development and precocity.

Organizational centres

Apical meristems and cambium are the chief organizational centres of the higher plants, but in the fertilized ovule it is clear that there is at least one organizational centre for the construction of the seed. It is the chalaza, which, morphologically, is the region of origin of the integuments and nucellus and which seems to act as the organizational centre for the developing seed after fertilization, comparable with the neuropore, or region of inflexion, of animal embryos. The basipetal growth of the Annonaceous ovule and seed, and the basipetal intercalation of the middle integument point to the antimicropylar end of the anatropous ovule, or the chalaza, as the pivotal region for development. This inference may be useful in physiological studies of seed development and of artificial development of fruits without pollination. It would seem that chalazal extracts, if such could be obtained, of fertilized ovules might be physiologically potent on embryonic or receptive tissue, such as stigmata. It is possible even that, from the study of the chalazal effects of seed development, chemical substances may be discovered which will lead to an understanding of the woody construction of mature seeds and so to the delignification of commercial drupes, e.g. *Mangifera*, as well as *Prunus*, and many other tropical trees for the discovery of seedless races of which breeding experiments will take too long.

Classification

The many genera of this difficult family are separated mainly by floral characters of macroscopic nature. The microscopic structure of the seed offers many new criteria, even to the extent of regrouping genera and parts of genera. Thus, *Canangium*, *Mezzettia* and *Xylopia Curtisii*, which are not particularly related in systematic works, have the peculiarity of the middle integument: and this character is absent from other species referred to *Xylopia*. *Desmos dasymaschala* has the testa structure of *Annona*, while *Desmos chinensis* and *D. dumosa* agree with *Xylopia caudata* and *X. malayana*. *Artabotrys* and *Mezzettia* have a layer of fibrous or prismatic cells perpendicular to the surface of the seed, immediately below the hypodermis of the outer integument, in contrast to the usual layer of longitudinal fibres. *Popowia tomentosa* has a parenchymatous (? pulpy) exterior to the testa as in *Xylopia fusca*, while *Popowia nervosa* agrees with *Polyalthia purpurea* in having a wholly fibrous testa. As regards the contents of the endosperm cells:

With starch	With oil (no starch)
Polyalthia purpurea	*Anaxagorea javanica*
Popowia nervosa	*Annona squamosa*
Desmos dumosa (and oil)	*Desmos chinensis* (very little starch)
D. dasymaschala (and oil)	
Xylopia malayana	*Xylopia fusca, X. caudata*
	Alphonsea elliptica
	Canangium odoratum
	Mezzettia leptopoda

Crystal cells in the hypodermis of the testa are characteristic also of *Annona squamosa*, *Desmos*, *Xylopia caudata* and *X. malayana*, and in the outer epidermis of the middle integument of *Canangium odoratum* and *Xylopia Curtisii*. They are absent from the other species which I studied.

Anthocyan occurs in the epidermal cells of the seeds of *Desmos dasymaschala* as in many species of *Xylopia*.

Oil-cells occur in *Anaxagorea javanica* (*i.i.*), *Annona squamosa* (nucellus), *Desmos* (nucellus), *Polyalthia purpurea* (*i.i.*), but are absent from the seeds of the other species which I studied.

Clearly, it will be necessary to tabulate these complicated seed characters in the endeavour to understand phyletic connexions.

Middle integument

As mentioned previously, I consider the middle integument to be a new feature of the seed developed as a result of extreme basipetal concentration of the growth of the ovule, after fertilization, at the antimicropylar end. In *Canangium* and *Xylopia*, which have also the aril, the middle integument becomes the fourth integument of the seed. Thus, I think that the middle integument, though mainly a post-fertilization feature like the aril, nevertheless has a different phyletic origin. One can hardly doubt that it is an indication of affinity between *Canangium*, *Mezzettia* and the section of *Xylopia* containing *X. Curtisii*.

SUMMARY

The microscopic structure of the seed is described in *Anaxagorea javanica*, *Annona squamosa*, *Alphonsea elliptica*, *Artabotrys odorata*, *Canangium odoratum*, *Desmos chinensis*, *D. dasymaschala* and *D. dumosa*, *Mezzettia leptopoda*, *Polyalthia purpurea*, *Popowia nervosa*, *P. tomentosa*, *Xylopia caudata*, *X. Curtisii*, *X. fusca* and *X. malayana*.

Annona and *Canangium* have a rudimentary aril.

Canangium, *Mezzettia* and *Xylopia Curtisii* have a *middle integument* developed as a post-fertilization feature on the sides of the ovule between the outer and inner integuments, and functioning as the testa. Thus, there can be four integuments: the outer (first), the inner (second), the aril (third) and the middle (fourth) integuments.

Eleven peculiarities of the Annonaceous seed are listed. The term *perichalazal* is used to describe the main peculiarity arising from the basipetal growth of the ovule and seed and resulting in the chalaza extending in a hoop round the seed with the integuments attached to it.

The term *transmedian longitudinal* is used to describe the section at right angles to the median longitudinal and passing through the extremities of the object. *Arilostome* is used to describe the false micropyle of *Annona*, caused by the rudimentary aril. *Subhilar tissue* refers to the mass of tissue between the hilum and the endosperm: it is developed into a conical, woody plug in many of the Annonaceous seeds. *Antiraphe* is used to indicate the part of the ovule or seed on the opposite side from the raphe.

The results of the study are taken to illustrate the morphological principles of *postponed development* (particularly the delay from pre- to post-fertilization), *transference of function* (from one tissue or cell-layer to another), *organizational centres* of development (as the

chalaza) and the importance of microscopic seed structure in the classification of tropical trees.

It is suggested that the aril may have been a third pulpy integument exterior to the other integuments of the megasporangium of the pro-Pteridosperm and now reduced to a post-fertilization relic. On the other hand, the middle integument is regarded as a new intercalary feature.

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AN EMBRYOLOGICAL STUDY OF *LOBELIA PYRAMIDALIS* WALL., WITH SPECIAL REFERENCE TO THE MECHANISM OF NUTRITION OF THE EMBRYO IN THE FAMILY LOBELIACEAE

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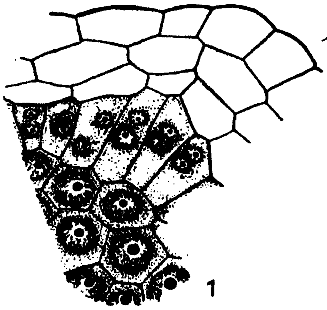
(With 52 figures in the text)

INTRODUCTION

Lobelia pyramidalis is a tall branched herb with linear lanceolate leaves bearing dense racemes of numerous purple or rose-coloured flowers. Some material of this plant was collected from Darjeeling at a height of about 7000 ft. and fixed in formalin-acetic-alcohol. Dehydration and embedding were done in the usual manner. Sections were cut at a thickness of 10–20 μ and stained in Heidenhain's iron-alum-haematoxylin with eosin or light green as a counterstain.



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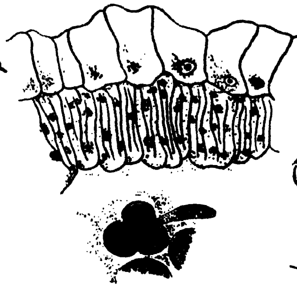

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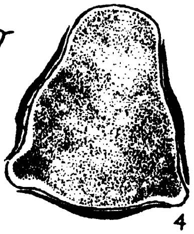

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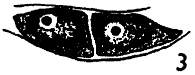

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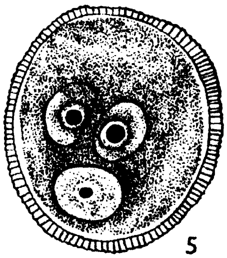

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Fig. 1. Portion of a cross-section of a young anther showing wall layers, binucleate tapetum and microspore mother cells. ×485.

Fig. 2. Germination of a pollen grain *in situ* within the anther loculus. Note male cells in the pollen tube. ×291.

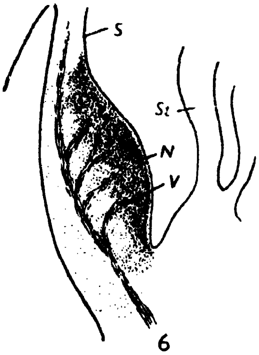
Fig. 3. A portion of the pollen tube enlarged to show the two male cells. ×970.

Fig. 4. Pollen grain showing three germ pores. Note that the pollen tube has emerged from the upper germ pore. ×970.

Fig. 5. Mature pollen grain showing the tube nucleus and the two male cells. ×970.

MICROSPORANGIUM AND MALE GAMETOPHYTE

The wall of the anther (Fig. 1) is made up of three layers of cells external to the tapetum. The outermost is the epidermis, and the next is the endothecium which acquires fibrous thickenings at maturity (Fig. 2). The single middle layer soon becomes flattened and crushed. The tapetal cells are uninucleate at first but later become binucleate. They are radially elongated and conspicuously vacuolated, and are of the glandular type.



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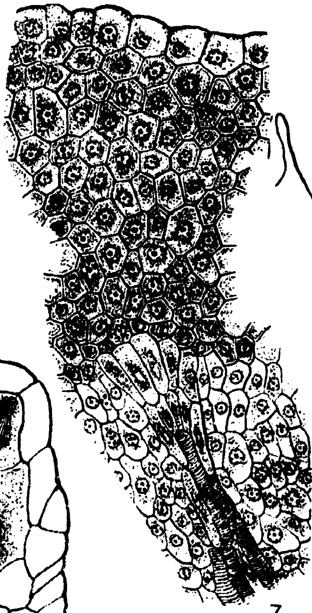

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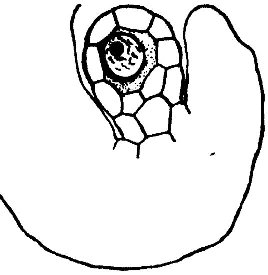

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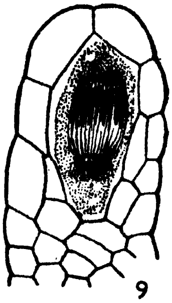

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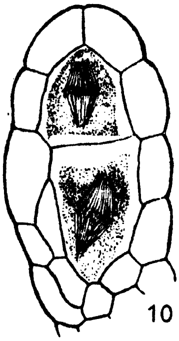

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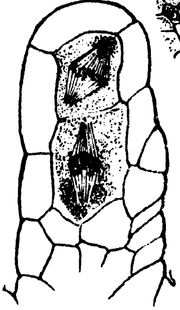

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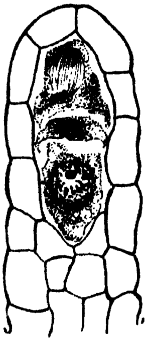

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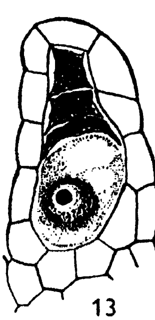

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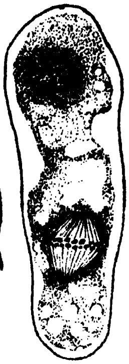

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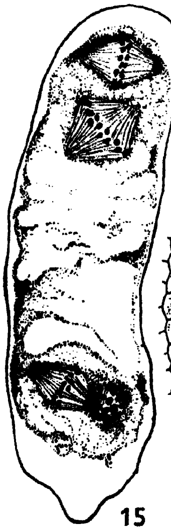

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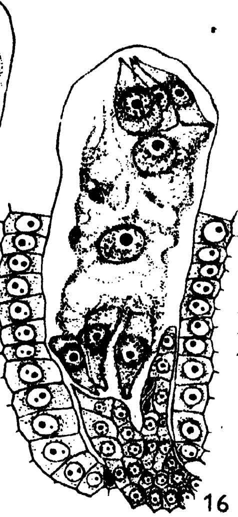

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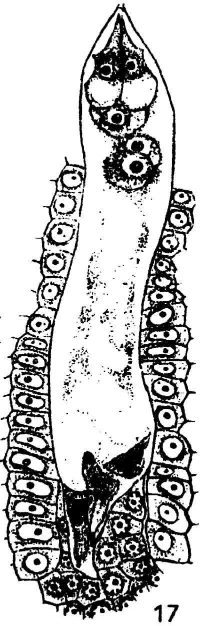

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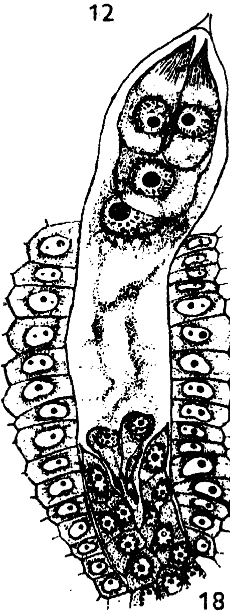

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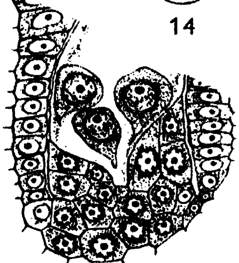

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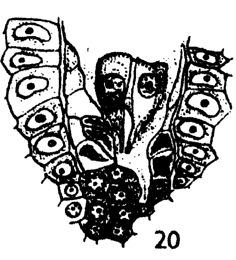

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The microspore mother cells undergo the usual reduction divisions and form tetrads of microspores. Quadripartition of the mother cells takes place by peripheral cleavage furrows and the microspores are arranged tetrahedrally. The mature pollen grain (Figs. 4, 5), which is provided with the usual exine and intine, contains a tube nucleus and two male cells as in other species of *Lobelia* (Kausik, 1938; Cooper, 1942; Kausik & Subramanyam, 1945*b*).

In a few loculi the pollen grains were found to have germinated *in situ*, and even the male cells had migrated into the germ tube (Figs. 2, 3). A similar precocious germination of the pollen grain has also been recorded in *L. trialata* (Kausik & Subramanyam, 1945*b*).

MEGASPORANGIUM AND FEMALE GAMETOPHYTE

The ovary is inferior, bicarpellary and syncarpous with an indefinite number of anatropous unitegmic ovules borne on a massive lobed placenta. Sparsely distributed pointed unicellular hairs are found on the outer wall of the ovary. Just above the ovary and within the whorl of stamens there is a conspicuous slightly lobed nectary which consists of glandular cells with conspicuous nuclei and dense cytoplasm (Figs. 6, 7). It receives branches of the vascular strand which passes up into the style. In *Dampiera stricta*, a member of the closely allied family Goodeniaceae (Brough, 1927), there are five well-developed [illegible]ries at the base of the style.

T[illegible] the po[illegible] at first as a conical outgrowth. The primordia of the integument app[illegible]lization [illegible]neously with the differentiation of the hypodermal archesporial cell (Fig. [illegible]) is f[illegible] functions directly as the megaspore mother cell. Tetrad formation (Figs. 9–12) takes place normally and the chalazal megaspore functions (Fig. 13). Sometimes, the upper dyad cell shows a belated division (Fig. 12), or the division is oblique (Fig. 11), as also recorded in *Cephalostigma Schimperi* (Kausik & Subramanyam, 1947*a*) and *Wahlenbergia gracilis* (Subramanyam, 1948). The functioning megaspore undergoes three successive divisions (Figs. 14–17) to produce an eight-nucleate embryo sac of the *Polygonum* type (Maheshwari, 1948).

During the growth of the embryo sac the cells of the nucellar epidermis are all destroyed, so that the mature embryo sac comes in direct contact with the innermost layer of the integument which becomes modified to form the endothelium (Figs. 16–18).

Legends to Figs. 6–20

Fig. 6. Longitudinal section of the nectary *N* situated at the base of the style *S* and within the stamen *St*. Note the strong vascular supply *V*. × 30.

Fig. 7. A portion of the nectary enlarged. × 291.

Fig. 8. Ovule showing megaspore mother cell. × 485.

Fig. 9. Telophase stage in megaspore mother cell. × 485.

Fig. 10. Dyad cells in division. × 970.

Fig. 11. Same. Note oblique division in the upper dyad cell. × 970.

Fig. 12. Linear tetrad of megaspores; upper dyad cell still in telophase stage. × 970.

Fig. 13. Functioning megaspore. × 970.

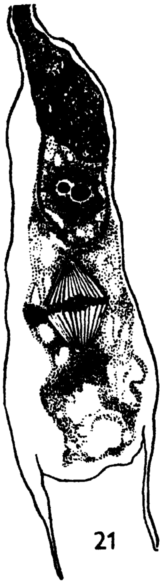
Figs. 14, 15. Second and third nuclear divisions in the formation of the embryo sac. × 970.

Fig. 16. Young embryo sac. Note process at the chalazal end of the embryo sac. × 679.

Fig. 17. Older stage showing degenerating antipodal cells. × 679.

Fig. 18. Mature embryo sac showing the filiform apparatus in the synergids. × 679.

Figs. 19, 20. Portions of two embryo sacs showing the chalazal process, the antipodal cells, the nutritive tissue of the chalaza and the integumentary tapetum. × 679.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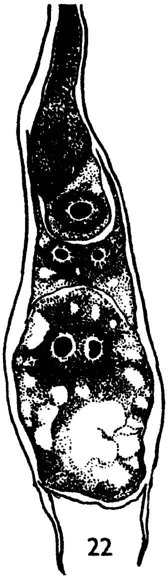
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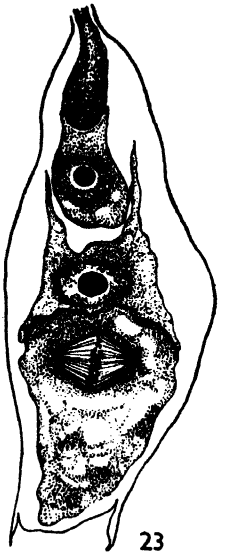
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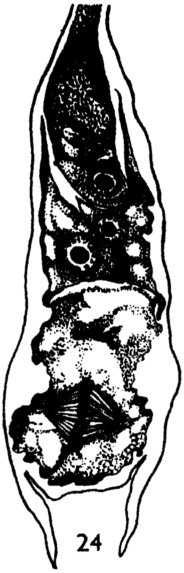
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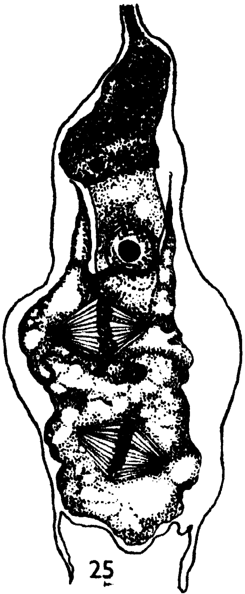
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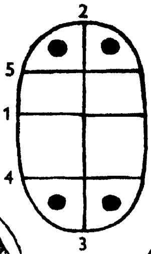

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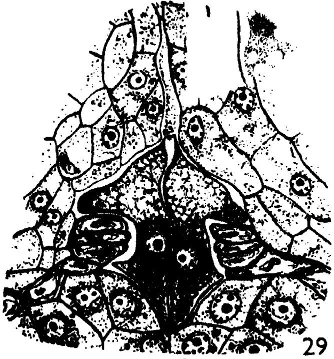
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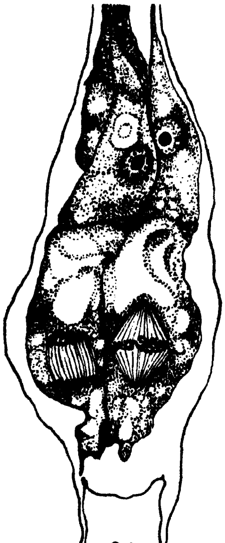
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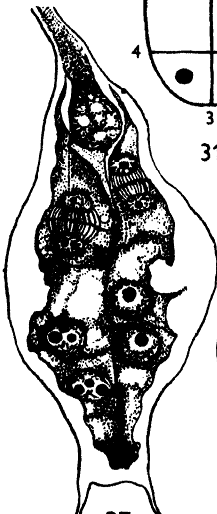
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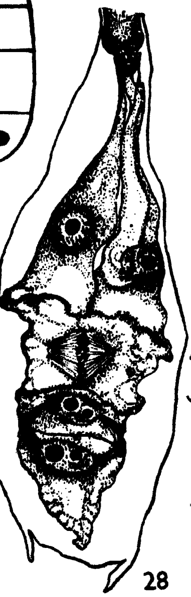
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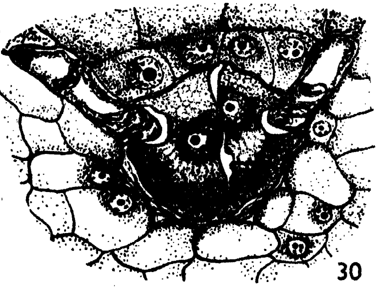
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Fig. 21. First division of the endosperm nucleus. × 679.

Figs. 22–27. Stages in the development of the endosperm and differentiation of the micropylar and the chalazal haustoria. Figs. 22–25, × 679 each; Figs. 26–27, × 485 each.

Fig. 28. A stage in the development of the endosperm according to the *Phyteuma* type. × 679.

Fig. 29. Two-celled micropylar haustorium in advanced stage. × 485.

Fig. 30. Two-celled chalazal haustorium in advanced stage. × 485.

Fig. 31. Diagram showing sequence of wall formation in endosperm.

The eight-nucleate embryo sac is long and tapers at both ends. The synergids have a pointed apical end and a broad vacuolate posterior portion (Fig. 17). They show characteristic hooks (Fig. 17) as in *Cephalostigma Schimperi* (Kausik & Subramanyam, 1947*a*) and *Wahlenbergia gracilis* (Subramanyam, 1948), and frequently show a filiform apparatus (Fig. 18) similar to that recorded by Cooper (1942) in *Lobelia cardinalis*. The egg is pear-shaped and is situated (Figs. 17, 18) between the two synergids. The two polar nuclei meet very near the egg and fuse to form the secondary nucleus just prior to fertilization (Figs. 16–18). As in other species of *Lobelia* (Kausik, 1935; Hewitt, 1939; and Kausik & Subramanyam, 1945*b*) and in *Cephalostigma Schimperi* (Kausik & Subramanyam, 1947*a*) of the closely allied family Campanulaceae, the antipodals are organized into definite cells whose lower ends are somewhat pointed (Figs. 16–20). They usually disorganize either before fertilization or at the time of fertilization. The only exception is *Lobelia cardinalis*, in which Cooper (1942) reports that the antipodal cells give rise to a chalazal haustorium. This is no doubt incorrect, as already pointed out by Maheshwari (1944*a*).

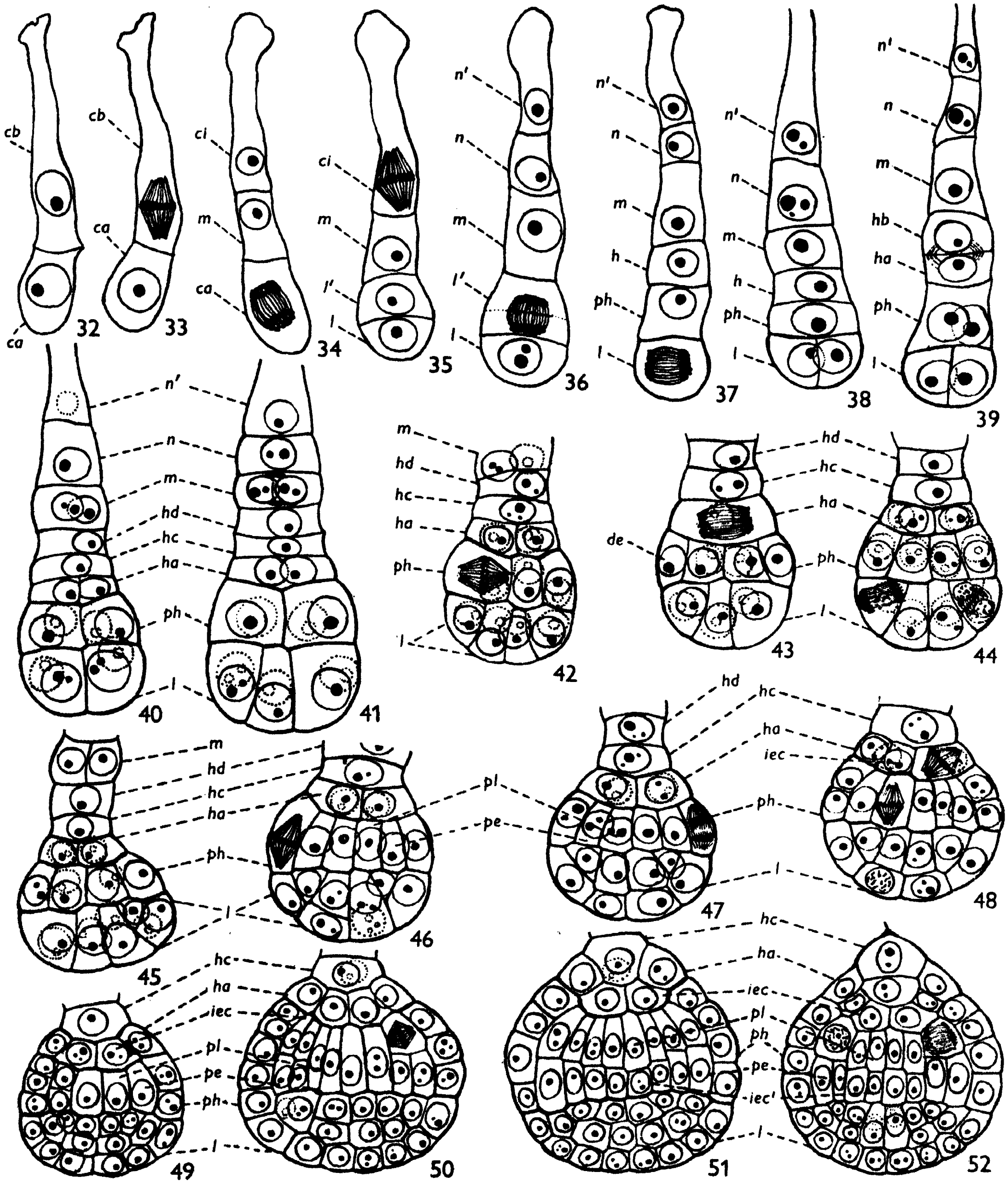
It is to be noted that the lower end of the embryo sac begins to grow past the antipodals (Figs. 16, 17) as a small haustorial process penetrating into the chalazal end (Figs. 16–20) (cf. *L. nicotianaefolia* studied by Kausik, 1938).

ENDOSPERM

When the pollen tube enters the embryo sac one of the synergids is destroyed. Double fertilization takes place normally. The division of the primary endosperm nucleus (Fig. 21) is followed by the laying down of a transverse wall resulting in a primary micropylar and a primary chalazal chamber. A vertical wall is then laid down in the primary micropylar chamber (Fig. 22) followed by a similar wall (Fig. 24) in the primary chalazal chamber. Sometimes the division in the primary chalazal chamber takes place first (Fig. 23), or both the vertical divisions are simultaneous (Fig. 25). In either case the endosperm is now made up of four cells, with two cells in each tier.

Transverse walls are now formed in each of the two tiers of the four-celled endosperm, first in the lower tier of cells (Fig. 26) as in *L. trialata* (Kausik & Subramanyam, 1945*b*), and then in the upper tier (Fig. 27); the result is an eight-celled endosperm made up of four tiers of two cells each. *L. amoena* (Hewitt, 1939), *L. trigona* (Maheshwari, 1944*b*) and *L. nicotianaefolia* (Kausik & Subramanyam, 1946) are slightly different in that transverse walls are first laid down in the upper tier and then in the lower.

At the eight-celled stage of the endosperm, the two cells of the upper tier develop into the micropylar haustorium and those of the lower into the chalazal haustorium. The two middle tiers form the main body of the endosperm. Thus the sequence of wall formation (Fig. 31) closely corresponds to the *Scutellaria* type of Schnarf (1931). A similar sequence of divisions has been reported in other species of *Lobelia* (Hewitt, 1939; Maheshwari, 1944*b*; Kausik & Subramanyam, 1945*b*, 1946). Fig. 28 shows a case where the formation of the vertical wall in the primary micropylar chamber was followed by a transverse wall in the primary chalazal chamber resulting in three tiers of cells, the upper consisting of two cells and the middle and lower of only one cell each. Of these, the lowest cell has nearly completed another transverse division and the middle cell is in process of a vertical division. If now the two uppermost cells were to divide transversely, the sequence of wall formation would correspond to the *Phyteuma* type (Rosén, 1932) described for



Figs. 32–52. Stages in the development of the embryo. Primary segmentation walls are indicated by thicker lines. ×485 each.

Lettering: *ca*, terminal cell of the two-celled embryo; *cb*, basal cell of the two-celled embryo; *m* and *ci*, cells derived from the basal cell *cb*; *n* and *n'*, daughter cells of *ci*; *l*, lower daughter cell of *ca*; *l'* upper daughter cell of *ca*; *ph* and *h*, daughter cells of *l'*; *ha* and *hb*, daughter cells of *h*; *hc* and *hd*, daughter cells of *hb*; *iec*, initials of radicle; *de*, dermatogen; *pe*, periblem; *pl*, plerome.

certain members of Campanulaceae. Other members of the Campanulaceae show the so-called *Codonopsis* type. It is interesting to note that these two types of development also occur in certain genera of Lobeliaceae (of Hutchinson) like *Lobelia*, *Pratia*, *Isotoma* and *Laurentia* (Rosén, 1932; Kausik & Subramanyam, 1945*a*).

ENDOSPERM HAUSTORIA IN THE MATURE SEED

The micropylar haustorium (Fig. 29) remains active for a long time. The two cells of which it is composed show a dense finely vacuolate cytoplasm, a large nucleus and a prominent hump. This haustorium absorbs food from the micropylar cells of the integument.

The distal ends of the two cells of the chalazal haustorium (Fig. 30) become extended and spread out. This haustorium ceases its activities earlier than the micropylar haustorium.

The endosperm cells lying between the two haustoria are densely packed with starch grains except in the region immediately around the developing embryo. Probably the latter secretes some enzymes which dissolves the starch. The seed coat is fairly rigid and hard on account of the numerous thickenings that are formed in the outermost layer of cells.

EMBRYO

The fertilized egg becomes rapidly elongated, and the first division is followed by the formation of a transverse wall (Fig. 32). Similar transverse walls are laid during subsequent divisions so that finally there results a long and slender pro-embryo with a number of cells (Figs. 33–37). The lowest cell, *l*, then divides by a vertical wall (Figs. 37–38), followed by the laying down of a similar vertical wall in the cell *ph* (Fig. 39). The four cells thus formed divide by a second set of vertical walls at right angles to the first set (Fig. 40). Meanwhile the third cell of the pro-embryo, *ha*, also divides by a vertical wall, and the fourth, *hb*, divides by a transverse wall to produce *hc* and *hd* (Fig. 40). The embryo proper is formed from the cells *l*, *ph*, *ha* and *hc*, and the rest of the cells of the pro-embryo constitute the suspensor.

At about the octant stage of the embryo the cell *m* undergoes a vertical division (Figs. 40–42, 45) as in *Lobelia amoena* (Hewitt, 1939), *L. trilata* (Kausik & Subramanyam, 1945*b*) and *Isotoma longiflora* (Kausik & Subramanyam, 1947*b*). At the same time oblique walls arise in the octants to form the dermatogen *de*, and after further divisions the periblem *pe* and plerome *pl* are also differentiated (Figs. 40–49). Divisions also take place in the third embryonal tier *ha*, giving rise to a semicircular group of cells (Figs. 48–52). The embryo is now large and spherical (Figs. 50–52) and further development takes place rapidly. The tier *l* now gives rise to the cotyledons and stem tip; *ph* to the hypocotyl; *ha* contributes to the periblem and a part of root cap; and *hc* to the remaining part of the root cap. The development thus corresponds to that described in other members of the Lobeliaceae (Kausik, 1935, 1938; Crete, 1938; Hewitt, 1939; Kausik & Subramanyam, 1945*b*, 1947*b*).

THE NUTRITION OF THE EMBRYO SAC AND EMBRYO

In all members of the Lobeliaceae, the initial nutritive requirements of the growing embryo sac are met by the cells of the integumentary tapetum. This layer extends considerably below the chalazal end of the embryo sac and thus encloses within its limits a part of the chalazal nutritive tissue. In *Lobelia trigona* (Kausik, 1935) the tapetal cells at the chalazal end of the embryo sac have denser contents than those situated at a higher level. Apparently they absorb food from the cells of the chalazal nutritive tissue and transport it to the embryo sac. But when the embryo is mature, the tapetal cells become thick-walled and impervious. Thus this layer has two separate functions during the maturation of the seed, at first nutritive and then protective.

The chalazal nutritive tissue already mentioned is made up of cells with dense cytoplasm and a prominent nucleus. Part of it is used up before fertilization and the rest soon after the formation of the chalazal haustorium.

The behaviour of the chalazal end of the embryo sac deserves mention. As stated before, it forms a process which grows beyond the antipodal cells and projects into the chalazal nutritive tissue forming a weak embryo-sac haustorium which is later replaced by the more aggressive endosperm haustorium. The antipodal cells with their pointed lower ends also seem to be adapted for the absorption and conduction of food to the upper part of the embryo sac. They begin to disorganize only when the embryo sac is ready for fertilization.

A nutritive tissue is also present in the micropylar region of the integument. It becomes conspicuous only when the micropylar haustorium is well differentiated. Both the micropylar and chalazal haustoria are aggressive structures, although usually the micropylar persists for a longer period than the chalazal. The embryo has a very long suspensor which pushes it into the cells of the endosperm. Possibly the suspensor also serves to facilitate the conduction of the food materials absorbed by the micropylar haustorium to the developing embryo.

The vascular supply of the ovule has been described by Cooper (1942) for *L. cardinalis.* A number of thin-walled elongated cells with dense cytoplasm and ovoid nuclei extend from the tip of the vascular strand to the chalazal end of the embryo sac. Further, some small parenchymatous cells intervene between these cells and the nutritive cells of the chalaza. According to Cooper (1942) they remain vacuolate until the ovule is fairly mature and probably function in the osmotic conduction of water and food materials from the vascular strand to the developing seed.

SUMMARY

The wall of the anther is composed of three layers of cells. The tapetal cells are binucleate, the endothecium becomes fibrous, and the middle layer becomes flattened and crushed. The pollen grains are three-celled and sometimes germinate *in situ.*

The ovary is inferior and bilocular with an indefinite number of anatropous unitegmic ovules borne on an axile placenta. At the base of the style and intrastaminal in position there is a nectary. The innermost layer of the integument forms an integumentary tapetum or endothelium.

Megasporogenesis proceeds normally, and the embryo sac is of the monosporic eight-nucleate type. The synergids are elongated and have the characteristic hook-like projections. Sometimes they also show a filiform apparatus. The antipodals are organized as definite cells which are pointed at their lower ends. The embryo sac develops a small process at the chalazal end which serves for the absorption of food from the nutritive cells of the chalaza. Double fertilization occurs.

The endosperm is cellular and its development corresponds to the *Scutellaria* type of Schnarf. One case showing the *Phyteuma* type of Rosén was also met with. The micro-

pylar haustorium is made up of two uninucleate cells and is more aggressive than the chalazal haustorium which is also two-celled.

Development of the embryo follows the *Solanad* type as in other members of Lobeliaceae.

The mature seed contains a large mass of endosperm. The outermost layer of the seed coat shows thickenings.

It gives me great pleasure to thank Prof. P. Maheshwari for revising the manuscript and for offering his valuable suggestions, to Dr L. N. Rao for his kind encouragement and interest, and to Mr G. Ghosh of Darjeeling for providing the material on which this study is based.

REFERENCES

BROUGH, P. (1927). Studies in the Goodeniaceae. I. The life history of *Dampiera stricta* (R.Br.). *Proc. Linn. Soc. N.S.W.* **52**, 471–98.

COOPER, G. O. (1942). Microsporogenesis and development of seed in *Lobelia cardinalis*. *Bot. Gaz.* **104**, 72–81.

CRETE, P. (1938). Embryogénie des Lobeliacées. Developpement de l'embryon chez le *Lobelia syphilitica* L. *C.R. Acad. Sci., Paris*, **207**, 177.

HEWITT, W. C. (1939). Seed development of *Lobelia amoena*. *J. Elisha Mitchel Sci. Soc.* **55**, 63–82.

KAUSIK, S. B. (1935). The life history of *Lobelia trigona* Roxb. with special reference to the nutrition of the embryo sac. *Proc. Ind. Acad. Sci.* B, **2**, 410–18.

KAUSIK, S. B. (1938). Gametogenesis and embryogeny in *Lobelia nicotianaefolia* Heyne. *J. Indian Bot. Soc.* **17**, 161–8.

KAUSIK, S. B. & SUBRAMANYAM, K. (1945*a*). An embryological study of *Isotoma longiflora* Presl. *Proc. Ind. Acad. Sci.* B, **21**, 269–78.

KAUSIK, S. B. & SUBRAMANYAM, K. (1945*b*). A contribution to the embryology of *Lobelia trialata* Buch-Ham. *J. Indian Bot. Soc.* **24**, 175–81.

KAUSIK, S. B. & SUBRAMANYAM, K. (1946). Development of endosperm in *Lobelia nicotianaefolia* Heyne. *Curr. Sci.* **15**, 78–9.

KAUSIK, S. B. & SUBRAMANYAM, K. (1947*a*). Embryology of *Cephalostigma Schimperi* Hochst. *Bot. Gaz.* **109**, 85–90.

KAUSIK, S. B. & SUBRAMANYAM, K. (1947*b*). Embryogeny of *Isotoma longiflora* Presl. *Proc. Ind. Acad. Sci.* B, **26**, 164–7.

MAHESHWARI, P. (1944*a*). The origin of the haustoria in the ovule of *Lobelia*. *J. Indian Bot. Soc.* **23**, 79–91.

MAHESHWARI, P. (1944*b*). The origin of the haustoria in the ovule of *Lobelia*. *Curr. Sci.* **13**, 186–7.

MAHESHWARI, P. (1948). The angiosperm embryo sac. *Bot. Rev.* **14**, 1–56.

ROSÉN, W. (1932). Zur Embryologie der Campanulaceen und Lobeliaceen. *Acta Hort. Göteborgs.* **7**, 31–42.

SCHNARF, K. (1931). *Vergleichende Embryologie der Angiospermen.* Berlin.

SUBRAMANYAM, K. (1948). A contribution to the embryology of *Wahlenbergia gracilis* Schrad. *Proc. Nat. Inst. Sci. India*, **14**, 359–66.

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STUDIES IN THE MORPHOGENESIS OF LEAVES

III. PRELIMINARY OBSERVATIONS ON VEGETATIVE GROWTH IN *LEMNA MINOR*

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AND E. J. WINTER*

(With 4 figures in the text)

INTRODUCTION

Earlier papers in this series (Ashby, 1948 *a*, *b*) refer to the fact that successive leaves on the stems of flowering plants are not all alike, but show regular changes, from node to node, in cell size and number and in leaf shape. This fact raises the problem as to whether these regular changes merely reflect changes in the external environment during development, or are due to internal changes which occur even in a constant external environment. This problem is being studied in this laboratory using *Ipomoea*. For a critical analysis, however, it is necessary to use a plant which can be grown under closely controlled environmental conditions, and in which the points of origin of successive leaves are as close together as possible. These conditions are satisfied by *Lemna minor*. Some unpublished observations on *Lemna*, made by two of us (E. A. and E. J. W.) eleven years ago, form the basis of our present investigations, and our combined data are used in this paper.

It is known from studies of populations of *Lemna minor* (Ashby, Bolas & Henderson, 1928; Ashby & Oxley, 1935) that when growth is unrestricted, the number of fronds increases exponentially with time. But these studies throw no light on the contribution made by each frond-meristem to the growth of the whole population. What is the expectation of life of a *Lemna* frond? Do fronds continue indefinitely to produce daughter fronds? Are the successive daughter fronds produced from one mother frond all alike in size and morphology? These questions have a direct bearing on the problems of heteroblastic development and physiological age which are the subject of the present series of papers; and they can be studied in *Lemna* with the assurance that environmental conditions are under control and morphological complexities are at a minimum.

Morphologists do not agree about the way to interpret the frond of *Lemna*, but for the purpose of this study it is unnecessary to hold an opinion as to what a *Lemna* frond is. Whether fronds are regarded as leaves (Goebel, 1891–3), or phyllodes (Arber, 1919), or cladodes (Hegelmaier, 1868), or shoots (Caldwell, 1899), they are morphological units of the same organism separated by intervals of time, and therefore subject to any process of senescence the organism may undergo.

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* Some of the observations recorded in this paper were made by two of us (E. A. and E. J. W.) in the University of Bristol in 1937. Our thanks are due to Prof. M. Skene for the facilities afforded to us at that time.

The adult frond of *Lemna* is approximately pear-shaped, with its acute end proximal to the mother frond. On either side of the proximal end, in the body of the frond, there is a 'pocket' from which daughter fronds are produced (Fig. 1 *a*, *b*). Daughter fronds do not appear simultaneously on both sides of the mother frond: they are produced alternately from one side and from the other, and clones of *Lemna* seem to be consistent in respect of the side of the mother frond from which the first daughter frond is produced. In *Lemna minor* (unlike *L. trisulca*) each mother frond produces more than two daughter fronds; successive fronds from the same pocket arise from almost contiguous meristematic zones (Fig. 1 *c*, *d*), so that daughter fronds, although considerably separated in time, are very little separated in space. Under the conditions of the experiments, a daughter frond became detached from the mother frond before the next daughter from the same pocket began to enlarge.

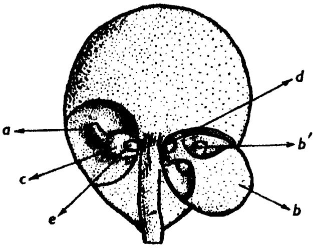

Fig. 1. Diagram of a frond of *Lemna minor*, from above, with flaps of pockets cut away. In the left pocket the first daughter frond has separated from the parent frond; only its stalk remains (*a*). Above *a* is the third daughter frond (*c*) just beginning to enlarge, and the fifth (*e*), still a primordium. In the right pocket is the half-grown second daughter frond (*b*), with its own first daughter frond (*b'*), and above the second daughter is the fourth daughter (*d*), still a primordium.

EXPERIMENTAL PROCEDURE

Experiments were carried out to obtain answers to the following questions: (i) How long does any one frond live? (ii) How many daughter fronds does each mother frond produce? (iii) What differences are there between successive daughter fronds from one mother frond? (iv) What is the relation between cell size and frond size during growth?

The experimental procedure was simple. Small colonies of *Lemna* were grown in constant conditions of light, temperature, and nutrient solution by a technique already fully described (Ashby & Oxley, 1935*). Fronds selected for observation were marked as soon as they appeared with specks of indian ink (which had been shown in preliminary trials to have no effect on growth rate). In earlier experiments one marked frond was distinguished from another by the position of the indian ink speck; in later experiments each marked frond occupied a numbered hole (1·5 cm. in diameter) in a perforated sheet of perspex, floated on the surface of the culture solution. The date of appearance and position of the daughter fronds from each marked mother frond were then recorded. Observations were made every 24 hr. Each daughter frond was recorded when it first appeared beyond the rim of the mother frond. Since the mother frond does not grow in width after its first daughter frond appears, these records are valid measures of the intervals between successive fronds. The side of origin of daughter fronds is described as follows: if a frond floats with its point of attachment to the mother frond toward the observer, then daughters which appear to the right are described as 'right fronds' (R.), and those to the left as 'left fronds' (L.).

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* Details of the technique are as follows. In all experiments Clark's (1926) culture solution was used. Its composition is 0·4 m.mol. Ca as $CaH_2(PO_4)_2$, 8·0 m.mol. K as KNO_3, 1·0 m.mol. Mg as $MgSO_4$; 0·01 m.mol. Fe as $FeCl_3$; to 1 l. of water. The pH was adjusted to 4·8. The light intensity in all experiments was 400 f.c., and the temperature was 26·5° C. (in 1937 experiments), and 25·5° C. (in 1948 experiments). In 1937 colonies were grown in continuous light, and in 1948 under a 14 hr. day.

VEGETATIVE LIFE HISTORY

The data from three experiments are summarized in Tables 1 and 2.

Table 1. *Mean length of life of fronds, with standard errors* (S.E.), *and mean intervals between successive daughter fronds*

Clone ...	I (1937), mean	II (1948)		III (1948)	
		Mean	S.E.	Mean	S.E.
Mean length of life of mother fronds (days)	42	41	0·93	36	1·30
Position of first daughter frond	L.	L.	—	R.	—
Mean interval (days) between daughter fronds:					
0–1	5	5·0	0·3	5·0	0·1
1–2	2	6·2	0·5	6·0	0·3
2–3	2·5	13·2	1·4	17·3	0·6
3–4	3	8·4	2·2	4·3	1·4
4–5	3	12·5	2·5	—	—
5–6	3	—	—	—	—
6–7	3	—	—	—	—
7–8	3·5	—	—	—	—
8–9	3·5	—	—	—	—
9–10	4·5	—	—	—	—
10–11	4·5	—	—	—	—
11–12	5·0	—	—	—	—

Table 2. *Distribution of number of daughters from mother fronds*

Percentage of fronds producing:	Clone II	Clone III
Only 1 daughter	0	0
Only 2 daughters	0	30·3
Only 3 daughters	11·1	42·4
Only 4 daughters	66·7	27·3
Only 5 daughters	22·2	0

Although these data are not extensive they lead to three simple conclusions, which are supported by numerous other experiments:

(*a*) *Lemna* fronds do not remain alive for long periods. Under the conditions of the experiments each frond lived for about 5–6 weeks. Natural death of a frond follows a gradual yellowing, which is readily distinguished from death due to other causes.

(*b*) During its life each frond gives rise to a limited number of daughter fronds, the number depending upon the clone of *Lemna* used. Each mother frond, together with its daughters, may therefore be regarded as a shoot of determinate growth, in which a limited number of nodes are separated in time, though not in space; and, broadly speaking, each daughter frond repeats its mother's history. The pattern of development shown in Fig. 1 may be interpreted, therefore, as a sympodial system of shoots, each shoot bearing a number of nodes corresponding to the mean number of daughters characteristic of the clone (Fig. 2).

(*c*) *Lemna minor*, already known to exist in physiologically different clones (White, 1936), exists also in morphologically different clones. There is some indication that the pattern of development may be changed by changing the environmental conditions, but confirmation of this must await further experiment.

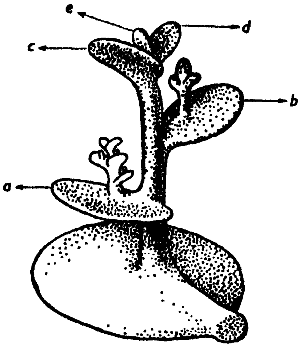

Fig. 2. Diagram of morphological interpretation of clone II of *Lemna minor*. The base of the model represents the mother frond, which bears five daughter fronds in succession at a meristematic zone here shown elongated into a shoot. Each daughter frond repeats the history of the mother frond. Growth is therefore sympodial. Lettering as in Fig. 1, except that *a* represents a frond, not the stalk of a frond which has become detached.

MORPHOLOGICAL DIFFERENCES BETWEEN SUCCESSIVE DAUGHTER FRONDS

In the course of observations on the vegetative life history of *Lemna* it was noticed that the areas of successive daughter fronds produced from one mother frond progressively diminished. It is this circumstance which makes the morphogenesis of *Lemna* relevant to work already published (Ashby, 1948*b*) on *Ipomoea*: for in *Lemna* such a trend as this in frond area is not likely to be due to changes in the environment, or to increasing distance from the source of nutrient supply, or to increasing differentiation of the plant body. Accordingly, it is reasonable to suppose that the trend of diminishing area is related either to some nutrient deficiency or to some process of ageing at the meristems of the mother frond; it is unlikely to be due to nutrient deficiency, because the *average* area of fronds in a colony does not diminish with time, but remains approximately constant. This indicates that late daughter fronds with sub-average area must in their turn give rise to early daughter fronds of more than average area. This behaviour has indeed been noticed and is now being thoroughly studied.

Data on the areas of successive daughters were collected as follows: while experiments on the life histories of clones II and III were in progress, daughters of the fronds under observation were segregated in separate holes in the floating perspex sheets. These daughter fronds were kept in culture for about 11 days, until they in turn had produced at least two daughter fronds: this was to ensure that they were fully grown. They were then fixed in formalin-acetic alcohol. For observation under the microscope the preserved fronds were prepared as follows: they were cleared in Eau-de-Javelle for about 3 hr., washed and dehydrated to 50% alcohol, left for 3 min. in a mixture of equal parts of 50% alcohol and glycerine jelly, and mounted in glycerine jelly. This treatment provides transparent mounts, in which every cell layer can be seen by focusing at different levels. Areas of the fronds were then determined by tracing micro-projections of them and measuring the areas of the tracings with a planimeter. The data are given in Fig. 3.

It is evident from Fig. 3 that in both clones there is a highly significant trend of diminishing area among successive daughter fronds from the same mother frond. It remained to determine whether the smaller areas are due to smaller cells in the fronds, or to fewer cell divisions; this was done by comparing the sizes of epidermal cells. It is impossible to estimate from random samples a reliable average cell size for the epidermis of a *Lemna* frond, because there are steep gradients of cell size from tip to base of the frond (see p. 379). Therefore three regions of the lower epidermis were chosen, which could be located regardless of its size and shape: one near the tip (*A*), one near the point of emergence of the root (*B*), and one near the base of the frond (*C*); all three just to one side of the midrib. Comparisons were made of the cell size from corresponding regions of successive fully grown fronds. The results are entered in Table 3. Clearly there are no significant differences in cell size between corresponding regions of first to third daughter

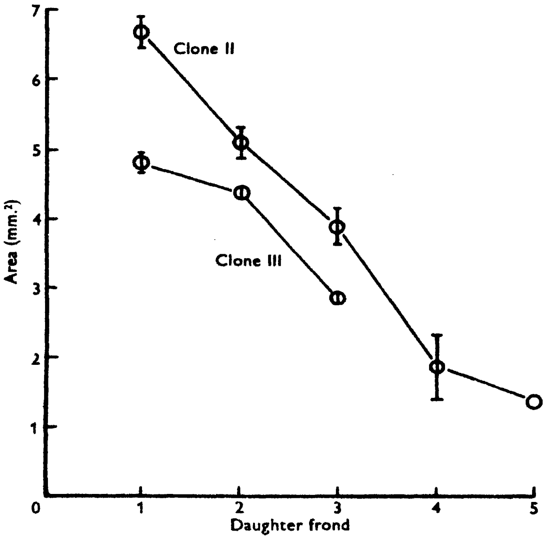

Fig. 3. Mean areas of fully grown daughter fronds in clones II and III. The vertical bars represent twice the standard errors of the values entered in the figure. There is no standard error for the fifth daughter frond of clone II.

Table 3. *Numbers of cells per mm.² from three regions of the lower epidermis of* Lemna minor, *in successive daughters*

(S.E. represents the standard errors of the means of the samples of fronds.)

	Clone II						Clone III	
Daughter...	1		2		3		1	3
Region	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	Mean
A	1728	58	1988	117	1728	99	2124	1959
B	1402	105	1325	79	1337	131	1894	1752
C	1254	57	1231	111	1319	86	1491	1349

fronds. Therefore the highly significant diminution in area of successive fronds (Fig. 3) must be attributed to a decrease in the number of cells per frond: this means either that there is a progressive diminution in the size of successive meristems, or successive meristems undergo progressively fewer cell divisions. There is no evident external cause for this trend, neither is it due to any evident 'position effect' of successive fronds. The third daughter frond develops in the same position as the first; but the first daughter breaks away from the parent frond while the third daughter is still a primordium, so that by the time the third daughter frond begins to enlarge there are no other developing fronds in the same pocket. The same applies to all subsequent daughters. The only evident factor which alters as successive daughter fronds appear is the age of the mother frond which produces them. For these reasons morphogenesis of *Lemna* is now being studied in relation to the ageing of the mother frond.

A NOTE ON CELL SIZE AND MORPHOGENESIS IN *LEMNA*

A preliminary study has been made of the changes in the size of epidermal cells which accompany the enlargement of a *Lemna* frond. The data for this study were obtained as follows.

Ninety fronds of different ages were fixed and cleared as described above (p. 377). The youngest fronds were little more than primordia and had to be squeezed out of the pockets of the parent fronds. Microprojections of the outlines of the cleared fronds were traced on paper. The length of each tracing from base to tip was measured. The fronds were then divided into nine approximately equal classes according to frond length, ranging from 1·00 to 3·30 mm. The purpose of this classification was to discover the mean length of epidermal cells corresponding to frond length at different stages of enlargement. It was found that, although epidermal cell size was approximately constant in transects at right angles to the long axis of the frond, it was by no means constant in transects parallel to the long axis; it is for this reason that reliable estimates of mean cell size cannot be made. The relation between frond length and cell length was accordingly worked out by taking samples of cell size from base to tip of every frond at intervals of 0·22 mm., along one side of the midrib. Samples were taken from the lower epidermis because it is free from stomata. Cell counts were made, using a square field 0·0169 mm.^{2} in area and a $\frac{1}{6}$ in. objective, by traversing cleared fronds with the aid of a mechanical stage. The number of whole cells in each sample field was counted, and a correction was added for the number of cells partly included in the field (see Ashby, 1948 *b*). Since the cells are, apart from convolutions on their radial walls, approximately isodiametric in surface section, the square roots of the reciprocals of these counts were used as estimates (for comparative purposes only) of cell length. The results are summarized in Table 4 and Fig. 4.*

The interrelationships between frond length, cell length, and position on the frond, are illustrated by a three-dimensional model constructed from smoothed data (Fig. 4). The following points are noteworthy:

(i) Even in the youngest fronds which were examined (fronds about one-third of their adult length) there is a steep gradient in epidermal cell size from base to tip (*a*): cells at the base of these fronds are small and actively dividing, whereas cells at the tip have reached almost their full size, their walls are convoluted, and fully developed stomata are present in the upper epidermis.

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* Clone III was used for these determinations. It is evident from Tables 1 and 2 (p. 376) that in a population first and second daughters greatly outnumber third and fourth daughters. The differences in cell number between successive daughter fronds therefore do not obscure the trend of the data.

(ii) In older fronds (*b*) the gradient in cell size is reversed. It appears that during enlargement of the frond a wave of cell expansion moves from the tip toward the base, and the region of maximum cell size in a mature frond is at *c*, about a quarter of the distance from base to tip.

Table 4. *Square roots of areas of cells in lower epidermis* (*mm.*)

(Estimate of average standard deviation of each entry, assuming variances are normally distributed = 12% of each mean. There are apparent coincidences among the entries because they are derived from the reciprocals of whole numbers.)

Distance along frond (mm.)	Class modes of length of frond (mm.)								
	1·00	1·29	1·57	1·86	2·14	2·43	2·71	3·00	3·30
0·22	0·007	0·008	0·007	0·012	0·015	0·023	0·023	0·023	0·023
0·44	0·010	0·012	0·015	0·020	0·024	0·025	0·026	0·026	0·025
0·66	0·018	0·017	0·016	0·024	0·024	0·026	0·027	0·027	0·025
0·88	0·019	0·018	0·020	0·019	0·024	0·025	0·026	0·025	0·025
1·10	0·018	0·019	0·020	0·024	0·023	0·025	0·026	0·026	0·023
1·32	—	0·020	0·020	0·023	0·022	0·024	0·024	0·024	0·023
1·54	—	—	0·019	0·022	0·022	0·024	0·023	0·023	0·022
1·76	—	—	0·020	0·021	0·022	0·023	0·023	0·022	0·022
1·98	—	—	—	0·020	0·021	0·022	0·022	0·022	0·022
2·20	—	—	—	—	0·020	0·021	0·021	0·022	0·022
2·42	—	—	—	—	—	0·020	0·021	0·021	0·021
2·64	—	—	—	—	—	0·020	0·020	0·021	0·021
2·86	—	—	—	—	—	—	0·020	0·020	0·021
3·08	—	—	—	—	—	—	—	0·019	0·020
3·30	—	—	—	—	—	—	—	—	0·020

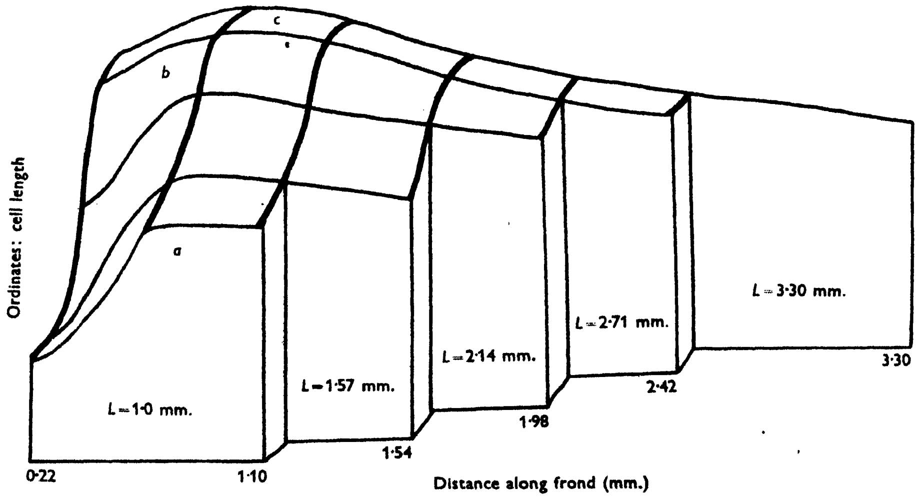

Fig. 4. Relationships between cell length and frond length in clone III. *Ordinates*: cell length; *left-to-right abscissae*: distance along fronds; *front-to-back abscissae*: stage of enlargement of fronds (in classes of frond length). *L* = class mode of frond length. An approximate estimate of the coefficient of variation of ordinates is c.v. = 12%; therefore differences exceeding about 24% of the ordinates are significant. *a* = smallest fronds, with small meristematic cells at the base and almost fully grown cells at the tip. *b* = large fronds, with largest cells near the base and smallest cells at the tip. *c* = region of maximum cell size in mature frond.

SUMMARY

Papers in this series are contributions to an analysis of the changes in leaf morphology from node to node on herbaceous plants. The present paper offers evidence that similar changes in morphology occur between successive fronds from the same meristem in *Lemna minor*.

Preliminary experiments establish four facts, namely, that in adequate conditions of light, temperature, and mineral nutrition: (i) *Lemna* fronds do not remain alive for more than 5–6 weeks; (ii) during its life each *Lemna* frond gives rise to a limited number of daughter fronds, the number being characteristic of the clone used; (iii) the areas of successive daughter fronds produced from one mother frond progressively diminish; (iv) this diminution in area is not associated with any reduction in epidermal cell size, but is due to the fact that there are fewer cells in late-formed than in early-formed daughters from the same mother frond.

The authors have pleasure in thanking Mr George Breckon for technical assistance and Mr E. H. Roberts for drawing Figs. 1 and 2.

REFERENCES

ARBER, A. (1919). Vegetative morphology of *Pistia* and the Lemnaceae. *Proc. Roy. Soc.* B, **91**, 96.

ASHBY, E. (1948*a*). Studies in the morphogenesis of leaves. I. *New Phytol.* **47**, 153.

ASHBY, E. (1948*b*). Studies in the morphogenesis of leaves. II. *New Phytol.* **47**, 177.

ASHBY, E., BOLAS, B. D. & HENDERSON, F. Y. (1928). The interaction of factors in the growth of *Lemna*. I. *Ann. Bot., Lond.*, **42**, 771.

ASHBY, E. & OXLEY, T. A. (1935). The interaction of factors in the growth of *Lemna*. VI. *Ann. Bot., Lond.*, **49**, 309.

CALDWELL, O. W. (1899). On the life history of *Lemna minor*. *Bot. Gaz.* **27**, 37.

CLARK, N. A (1926). Plant growth promoting substances, hydrogen ion concentration, and the reproduction of *Lemna*. *Plant Physiol.* **1**, 273.

GOEBEL, K. (1891–1893). *Pflanzenbiologische Schilderungen.* Teil II. Marburg.

HEGELMAIER, F. (1868). *Die Lemnaceen.* Leipzig.

WHITE, H. L. (1936). The interaction of factors in the growth of *Lemna*. IX. *Ann. Bot., Lond.*, **50**, 827.

WINTER, E. J. (1937). Growth of *Lemna minor*. *Nature, Lond.*, **139**, 1070.

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THE BASIDIOMYCETES OF SKOKHOLM ISLAND

II. GENETICAL IMPLICATIONS OF SPORE MEASUREMENTS IN TWO AGARICS

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1. Introduction

Observations made on the Basidiomycete flora of Skokholm Island on the west coast of Pembrokeshire during September 1947, and again in the same month of 1948, led me to suspect that some species of higher fungi found there might not be interchanging genes freely with the mainland populations; with a view to obtaining more precise evidence on this point, the studies reported in this paper were undertaken.

2. Theory

For reasons which will now be given, it seemed likely that a statistical analysis of spore measurements might show up any effect of this kind which was marked enough to attract attention.

The phenotypic characters of a basidiospore may be determined by four possible classes of factors; first, filial factors, by which is meant those reflecting the genotype of the spore itself; secondly, maternal factors, reflecting the genotype of the basidium (in the case of agarics, of the basidiocarp and ultimately the mycelium from which it springs); thirdly, environmental factors; and lastly, random factors, by which is meant those whose origin cannot with existing technique or knowledge be discerned. Of these four, the last class cannot be taken into quantitative consideration. Maternal and environmental factors may affect differences between the spores of one basidiocarp and another, but can hardly account for the statistical dispersion of any spore character among the spores produced by a single fungus, unless the hymenium is biseriate; it is possible, indeed, that there may exist a microclimatic difference between the edge, blade, and base of the lamellae, which might occasion a difference among the spores, and differences in nutrition between these sites might also do this, but such factors are not likely to alter in their incidence from one locality to another, nor will they be much affected by such relatively minor structural differences as are found within one species of agaric.

In considering the effect of filial factors upon spore characters, we must remember that the spores are, in most agarics, haploid; if therefore the parent fungus is heterozygous for, say, one pair of alleles affecting spore length, then the spores will fall into two equal classes, within each of which the lengths will be distributed about a different mean. If, apart from this one pair, all the genes affecting spore length have only small effects, and are numerous, they, with the random factors (and maybe those arising from lamellar location), will produce a roughly normal distribution within each class. In that case the overall distribution of spore length will be non-normal; if the means are sufficiently far apart, there will be a bimodal distribution, but more likely there will be no more than a slight separation, detectable as a *negative value for the fourth cumulant*. If, however, the sampling of the population is by means of a spore print, there may often be a complication, due to the correlation of the character studied with the rate of maturation of the spores; if one of the length classes is slower to ripen than the other, then that class will be under-represented in the spore print, and the observed distribution will be skew. The same will happen if the variances of the two classes are different; in either case the *third cumulant* will be affected, and the fourth cumulant will be in some instances positive rather than negative and therefore of less interest.

If, on the other hand, the fungus is heterozygous for several pairs of alleles having individually detectable effects on spore length, the various length classes resulting will combine to give a distribution which will be unlikely to be distinguishable from the normal; the same will happen if the fungus is homozygous for all the major factors for length, but in this case the *variance will be reduced* in comparison with the former case. It is not to be expected that the variance will be significantly reduced so long as there remains heterozygosity even for one pair of alleles.

Any barrier to the free interchange of genes between a smaller and a larger population will lead, if the smaller population is not itself too large, to a certain measure of inbreeding; this will be revealed in time by a decrease in the average heterozygosity of the population. The occurrence of genetic isolation in a population of heterothallic agarics on an island may very well be revealed, therefore, by a statistical analysis of suitable spore characters, since we have reason to expect that from their cumulants three possible levels of heterozygosity may be distinguishable. Outside these three levels there may be further statistical effects of less magnitude; thus, continued inbreeding may reduce the residual variance within each length class, and thus both reduce the general variance and make the fourth cumulant increasingly negative (if there is no skewness) for a given separation between the means, or, alternatively, make detectable by the higher cumulants a smaller separation. The smaller the variance relative to the higher cumulants, the more confidently may we infer inbreeding.

3. TECHNIQUE

To see whether these effects could be demonstrated, spore prints were taken from several specimens of two species of agarics collected from Skokholm Island, and from climatically comparable localities in the adjacent mainland parishes of Dale and Marloes, and (in one instance) from the small island of Gateholm, which is very close to the mainland and accessible dry-shod below half-tide. The species selected were *Panaeolus campanulatus* (L.) Fr., and *Leptonia solstitialis* Fr.*; these two are well separated taxonomically, readily available at the season of the year when the collecting was done, similar in their ecological relations but of contrasting habits of growth.

The characters selected for measurement were length and breadth of spores; details of these measurements will be given under the separate species. Measurements were made with an eyepiece micrometer using a $\frac{1}{12}$ in. oil-immersion objective, a 6 × eyepiece, and a tube length of 23 cm., giving a magnification of about 1300 diam. The length and breadth of each spore measured were recorded to the nearest odd multiple of one-quarter of the smallest micrometer divisions; that is to say, when one extremity of the spore was brought into optical coincidence with one scale division, judgement was made as to which marked division the other extremity was nearest to it, and to which side of it it lay; if, for example, one end lay on the mark 5·0 and the other lay just beyond 6·1, the length was given as 45 units, but if it were nearer to 6·2 it was recorded as 47. In order to avoid bias in sampling and repeated measurements of one spore, the microscope stage was moved in one direction only, and every spore encountered in the central part of the field was measured unless the two ends (or sides) could not be brought to focus simultaneously. Occasional spores were also passed over on account of manifest abnormality of shape or pigmentation, and on account of Brownian movement which sometimes made accurate measurement impossible. Rejection of spores which did not lie flat may perhaps have introduced a bias, but for the purposes of accurate measurement this was inevitable, and in any case the bias would be systematic and irrelevant to the analyses made.

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* From a description and a spore print Mr A. A. Pearson has suggested to me that this may be *Leptonia fulviceps* Rom. or *Nolanea papillata* Bres.

4. RESULTS WITH *LEPTONIA SOLSTITIALIS* FR.

This agaric is locally not uncommon in west Pembrokeshire; it grows in grassland communities and tolerates moderate exposure to sea spray. It is of low growth, and the pileus expands at or very often below the level of the surrounding herbage, so that a large proportion of the spores must be brought to rest on the grass quite close to the parent fungus. It is usual to find two or three fungi growing close together, presumably being sprung from one mycelium, but they do not form obvious rings. The spores are difficult to measure, being typically of very irregular form, roughly polygonal on optical section, with the angles often protuberant; it was found, however, that a long axis could be defined as being the greatest length measured from the middle of a rather well-marked plane face adjacent to the apiculus, which is large and conical. Consequently spores in which the apiculus could not be seen as they lay on the slide were rejected from the sample. The breadth was taken as the greatest length at right angles to the above-defined long axis; but, in measuring this, marked protuberances at the angles, which often added as much as a quarter to the whole breadth, were ignored.

Samples were taken from two fungi only; one from a south-facing slope on Dale Point, about 2 chains from the cliff edge, the other from the eastern part of Skokholm Island, about a furlong from the sea.

Table 1. *Spore statistics of* Leptonia solstitialis

Specimen	Length					Breadth				
	Mean	Variance	g_1^{-2}	g_3	g_4	Mean	Variance	g_1^{-2}	g_3	g_4
48D. *2b*	37·90	8·596	0·00598	+0·100	−0·362	23·72	2·335	0·00433	+0·080	**+0·074**
48S. 1	36·08	5·125	**0·00394**	+0·133	−0·785	24·72	1·800	**0·00294**	−0·071	**−0·113**

N.B. The units of calculation are equal to 0·275 μ. Figures for g_3 and g_4 which differ from zero with 5 % significance are put in **heavy type**, and if they reach 1 % significance they are *underlined*. Figures for g_1^{-2} are similarly treated when they differ from those obtaining for mainland specimens. These conventions are observed in all tables.

The results of the statistical analysis were as given in Table 1. The statistics tabulated are defined in terms of the cumulants k as follows: the mean and variance are of course k_1 and k_2 respectively; g_1^{-2} is the relative variance, k_2/k_1^2, which was taken as the basis of comparison rather than the absolute variance because it is likely that variations due to most factors will be proportional to the total length; g_3 is taken as $k_3/k_2^{1\frac{1}{2}}$, and g_4 is k_4/k_2^2. Each statistic is based, unless otherwise mentioned, on a sample of 100 spores, so that the sampling variances of g_3 and g_4 are the same throughout the series. The standard deviations come out as: for g_3 0·241, and for g_4 0·478. The difference between two estimates of g_1^{-2} may be taken as significant at the 5% level when their ratio is 1·45 and at the 1% level when it is 1·65 (approximate).

Since these data relate to two specimens only, it is unsafe to base any far-reaching conclusions on them, but it will be observed that both measurements in both specimens are within the limits of reasonable expectation normally distributed, and that the relative variances of both are significantly less in the Skokholm specimen than in the one from the mainland. So far as they go therefore these figures suggest that the former is the more homozygous for minor filial factors affecting spore size.

5. RESULTS WITH *PANAEOLUS CAMPANULATUS* (L.) FR.

This species is abundant in all parts of Britain; it affects a similar habitat to *Leptonia solstitialis*, and though it shows a preference for the richer types of pasture, it does not grow in direct association with the dung of animals. On Skokholm it is found chiefly in the eastern half of the island, like *L. solstitialis*, but is more abundant; this is probably because of the exposure of the western parts to salt spray, but neither species is among the most sensitive in this respect. *Panaeolus campanulatus* is taller in habit than the *Leptonia*, and its pileus generally opens an inch or two above the level of the grass, except in very rank vegetation, of which it is more tolerant; one would therefore suppose that its dispersal would be mainly anemophilous. The spores are easy to measure, and generally have a small slightly unsymmetrical apiculus at one end and a rather prominent papilla perforated by the germ pore at the other, but sometimes there is no apiculus and the prominence of the germ pore is very variable. In any case the length was always taken as the extreme end-to-end measurement, and the breadth as the greatest distance normal to the long axis; spores were rejected from measurement if either papilla or apiculus were seen otherwise than in optical profile and simultaneously in focus. It seemed that an appreciable part of the length variance was due to the variable development of the papilla, though the latter by itself was too small for accurate measurement; if this is so it implies that the two measurements made are more likely to be affected by different filial factors than if both are expressions of a single character of spore size, and thus that we may to some extent treat the two sets of statistics as independent; as we shall see, however, there are indications that they are fairly closely correlated. *Panaeolus campanulatus* has a multiseriate hymenium, which probably accounts for a part of the 'major factors' referred to below.

Table 2. *Spore measurements of* Panaeolus campanulatus *from Dale and Marloes*

Specimen	Length					Breadth				
	Mean	Variance	g_1^{-2}	g_3	g_4	Mean	Variance	g_1^{-2}	g_3	g_4
Pc. 48 D. 2*a*	40·58	8·27	0·00502	+0·274	−0·216	24·36	3·06	0·00517	+0·280	+0·506
Pc. 48 D. 2*b**	40·04	8·51	0·00531	**+0·445**	**+0·912**	24·36	5·01	0·00851	+0·280	−0·117
Pc. 48 D. 3	38·06	8·85	0·00611	+0·345	+0·166	24·36	4·11	0·00693	+0·149	−0·359
Pc. 48 D. Mean	39·58	8·54	0·00543	**+0·355**	+0·287	24·33	4·06	0·00685	+0·164	+0·010

* The figures for this specimen are based on 200 spores.

These three specimens ought to be regarded as at least potentially members of a single breeding population; the first two were collected within a few yards of each other, and one might therefore suppose that they would belong to one mycelium and would therefore be genetically identical, but from their statistics either this is not so, or else this one mycelium possesses more than two genetically different sets of nuclei (a thing which in the Basidiomycetes is possible through vegetative interchange of nuclei between two originally separate mycelia). The third specimen, Pc. 48 D. 3, was collected about 2 miles away in the parish of Marloes on the cliff edge, whereas Pc. 48 D. 2 came from the Isthmus of Dale; the agreement in the statistics between Pc. 48 D. 2*a* and Pc. 48 D. 3 is very close notwithstanding. Although one of the g_3 values for length is significantly greater than zero while the others are not, there are no significant differences among the three specimens, and all may be samples from a population having a slightly skew distribution of spore length; that being so, the differences among the g_4 values are less informative. In regard to breadth the differences in g_1^{-2} between Pc. 48 D. 2*a* and *b* are significant (just 1 %); we have, nevertheless, used the pooled value for comparison with the Skokholm figures, though the mainland population is evidently not quite as homogeneous as one might wish; the differences suggest that Pc. 48 D. 2*b* is more heterozygous than the other two in respect of major filial factors.

Table 3. *Spore measurements of* Panaeolus campanulatus *from Skokholm Island*

Specimen	Length					Breadth				
	Mean	Variance	g_1^{-2}	g_3	g_4	Mean	Variance	g_1^{-2}	g_3	g_4
Pc. 48 S. 1	48·90	7·55	**0·00316**	+0·259	−0·218	31·12	5·32	0·00549	+0·325	−0·267
Pc. 48 S. 2	50·48	8·45	**0·00332**	−0·339	−0·264	29·22	3·71	**0·00434**	**−0·501**	−0·279
Pc. 48 S. 4	38·98	8·77	0·00577	+0·132	−0·246	24·66	4·21	0·00692	+0·051	−0·673
Pc. 48 S. 5	43·22	9·61	0·00514	**+0·477**	−0·277	23·90	5·73	0·01004	**+0·838**	**+1·896**
Pc. 48 S. 6	48·12	14·49	0·00625	+0·323	−0·005	31·88	10·75	0·01031	**−0·469**	−0·173
Pc. 48 S. 8	41·02	8·38	0·00498	+0·262	−0·479	24·33	3·63	0·00613	**+1·921**	+0·370
Pc. 48 S. 9	46·82	6·55	0·00299	−0·271	−0·183	27·88	5·28	0·00682	−0·115	**−1·198**
Pc. 48 S. 15*a*	44·08	5·21	**0·00268**	−0·392	−0·444	26·90	1·89	**0·00261**	+0·064	−0·842
Pc. 48 S. Mean	**45·20**	8·63	0·00429	+0·056	**−0·264**	**27·49**	5·03	0·00658	+0·264	+0·146

In Table 3, except in the last row, figures for g_3 and g_4 are accounted significant if they differ sufficiently from zero, while in the case of g_1^{-2} significance is attached to difference from the corresponding pooled figures from Table 2. This is tested by a modified variance ratio method, on the grounds that the sampling variance attached to the means are much less than those attaching to the variances; in order to make some allowance for the heterogeneity attaching to the estimate of g_1^{-2} for breadth in Table 2, this has been assigned only 24 degrees of freedom instead of 399, which value makes equal the levels of 5 % significance between the *t*-test and the variance-ratio test. In the row giving the mean figures, the means, as well as all the *g* statistics, have been compared directly with the last row of Table 2.

In regard to length, all the specimens, except Pc. 48 S. 5, which just reaches significance in g_3, give a near-normal distribution which, however, is seen to be significantly on the flat side from the mean figure for g_4. This suggests that the population is homozygous for the major length factors such as affect Pc. 48 D. 2*b*, even though there is some heterozygosity for secondary factors. Four of the eight also show a significantly diminished relative variance, suggesting a greater degree of homozygosity for minor length factors, while of the other four three exhibit anomalies in the breadth distribution which render interpretation of the length statistics uncertain.

In breadth, there is a non-normal distribution in all but three, accompanied in the case of Pc. 48S. 2 by a reduced variance, implying that the number of factors affecting spore breadth has been diminished; in no case is the relative variance significantly increased, as it should be if there were increased heterozygosity as compared with the mainland. Of the eight specimens, only Pc. 48S. 4 could reasonably be a representative of the mainland population as judged from Table 2. The means of both measurements are, furthermore, very significantly greater in the insular population.

6. DISCUSSION

It is claimed that the results presented above afford evidence that, compared with the corresponding mainland populations, there is a significant measure of inbreeding, that is to say, a significantly less degree of heterozygosity in respect of certain loci, in the populations of *Panaeolus campanulatus* and *Leptonia solstitialis* found on Skokholm Island. If these species are mainly disseminated by atmospheric currents, as is usually assumed to be the case with the agarics, this could scarcely occur, for by this means spores could be carried to and from the island as easily as over a like distance between different parts of the mainland. It might be argued that the prevailing winds, during the season of fructification of our species, are south-westerly, and thereby unsuited to carry spores to the island which lies south and west of adjacent coasts; but at this season there are still a few easterly winds, and frequent days sufficiently calm to allow of the development of alternating land and sea breezes which, on account of their circulating character, are very well suited to effect a two-way transport, if an adequate number of viable spores were available to be caught up. This process must, indeed, frequently occur; but our results indicate that landwise transport, presumably by terrestrial animals of some kind, must play at least a part comparable with wind-borne spores, else there would be no impediment to the interchange of genes sufficient to bring about such differences between the two populations as we have found.

In assessing the magnitude of these effects, the island of Gateholm is useful; being so near to the mainland, separated by less than a furlong of littoral, its populations of agarics of like habit to those we are studying could scarcely show any isolative effect, unless anemophilous methods were distinctly less effective than transport by animals; but since to these the island is apparently difficult of access (except to *Homo* which is unsuitable as a vector) it might show as much isolation as Skokholm if dispersal were mainly zoophilous. Furthermore, its area being so small (about a tenth of that of Skokholm), one might expect it to develop an even greater degree of inbreeding. One specimen of *Panaeolus campanulatus* was collected on Gateholm, three days before the Skokholm collections and on the same day as the Dale and Marloes ones; Table 4 shows the statistics calculated in measuring a sample of 100 spores from it.

Table 4. *Spore measurements of* Panaeolus campanulatus *from Gateholm Island*

Specimen	Length					Breadth				
	Mean	Variance	g_1^{-2}	g_3	g_4	Mean	Variance	g_1^{-2}	g_3	g_4
Pc. 48G. 1	$\underline{52{\cdot}67}$	7·82	$\underline{0{\cdot}00282}$	−0·215	−0·104	$\underline{27{\cdot}28}$	2·85	$\underline{0{\cdot}00381}$	+0·528	−0·179

In both measurements, the statistics of this specimen conform to the Skokholm pattern rather than to the mainland one; in length, the relative variance is very significantly reduced, while the distribution remains insignificantly non-normal, and in breadth a diminished g_1^{-2} is accompanied by a significant skewness. The mean spore length is very significantly greater than both the mainland and the Skokholm means, and the mean breadth is greater than the mainland one. One cannot assert, however, that there is any evidence of specially severe inbreeding. This result seems quite inconsistent with the hypothesis that wind plays an appreciable part in spore dispersal of these fungi over short distances; for if that were the case the Gateholm population should be a part of the mainland one, and its statistics ought to lie within the limits of those of Table 2. As it is it differs at least in g_1^{-2} and g_3 in length (the latter differs from +0·355, but not from zero, at the 1% level) by more than can reasonably be expected. And if this applies to *Panaeolus*, it is likely to apply with the more force to *Leptonia* as being less adapted by its structure to an anemophilous dispersal.

Further to follow up these indications, it is intended to apply the same technique to other species of like habit, such as *Marasmius oreades*; and for comparison, species more likely to be anemophilous, such as the Lycoperdacei, ought to be examined. If it be accepted that terrestrial animals play a major part in the dissemination of some agarics, the questions arise: Which species are mainly concerned, and How do they perform this function? Owing to the high intensity of observation maintained during the summer months on Skokholm Island, it is likely that sufficient material to answer these questions may be forthcoming. It may also be asked: If an appreciable degree of genetic isolation obtains among these organisms, might they not be undergoing speciation? There is no indication yet that any kind of a geographic race or subspecies of any of the agarics exists on the island; but there certainly do seem to be a number of unusual variants present, which in a larger or less isolated population would have been eliminated, out of which by selection such a race might in time evolve. In the case of *Panaeolus campanulatus* there are forms distinguished by an unusually flat pileus, by a cracked or excoriated pellicle, by a pale-coloured stem, as well as the large spores attested above for 48S. 1, 2, 6 and 9, and 48G. 1; but these various characters do not seem to be much correlated with one another (see Table 5).

Table 5. *Aberrant characters among certain specimens of* Panaeolus campanulatus *from Skokholm and Gateholm*

Specimen	Flat pileus	Cracked pellicle	Pale stem	Large spores
Pc. 48S. 1	−	−	−	++
Pc. 48S. 2	−	−	−	+++
Pc. 48S. 4	−	−	−	−
Pc. 48S. 5	−	−	−	+
Pc. 48S. 6	−	++	−	++
Pc. 48S. 8	−	+	+	−
Pc. 48S. 9	+	++	−	++
Pc. 48S. 15*a*	−	++	+	+
Pc. 48G. 1	−	−	−	+++

However, it may well be that this species has entered on the first stage of speciation, and it is not impossible that further progress in this direction may have been made by other agarics on the island; that sufficient time has elapsed for this to happen is indicated by the existence of a local subspecies of *Microtus agrestis* on the neighbouring but geologically younger island of Scomer. In any case, it seems that a rich field exists here for the study of population dynamics and the long-term aspects of general ecology.

7. SUMMARY

The probable effects of various degrees of inbreeding upon the distribution of measurable spore characters in agarics are discussed; it is concluded that in favourable cases a comparison of the variance and higher cumulants of such distributions, taken from samples of spores from members of different populations, might provide evidence of differences in the level of inbreeding if there were any.

Methods of measuring the spores of *Panaeolus campanulatus* and *Leptonia solstitialis* are described. The application of statistical analysis to the figures thus obtained, from specimens collected from Skokholm and Gateholm Islands and the neighbouring mainland coast, is described, and the results tabulated.

It is concluded that in both species there is evidence that the insular populations are more highly inbred than those occurring on the mainland; and therefore that some barrier exists to the free interchange of genes across the intervening sea. It is suggested that this may result from the part played by terrestrial animals in the dissemination of these agarics being greater than has been commonly supposed.

The extent to which such isolation may result in the appearance of insular subspecies is discussed, and suggestions are made for further work on the problems raised.

I wish to express my indebtedness to the staff of the Council for the Promotion of Field Studies, at Dale Fort and Skokholm Island, for much personal help, information, and facilities. I also thank Prof. R. A. Fisher, F.R.S., for helpful discussion and criticism, and Mr A. A. Pearson for his comments on the spores of *Leptonia solstitialis* (if such it be).

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PHYSIOLOGICAL STUDIES ON ACID METABOLISM IN GREEN PLANTS

I. CO_2 FIXATION AND CO_2 LIBERATION IN CRASSULACEAN ACID METABOLISM

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CONTENTS

PAGE
Introduction 390
I. Acidification and deacidification in Crassulacean acid metabolism 392
  A. Peculiarities of Crassulacean acid metabolism 392
  B. Acidification and deacidification as determined by the relative rates of the production and consumption of acids 392
  C. Acid production as resulting from glycolysis and CO_2 fixation . . . 393
  D. The reduction in titratable acidity through the consumption of vegetable acids 395
  E. Previous hypotheses to account for diurnal variations in titratable acidity with special reference to Wolf's hypothesis 396
II. Evidence bearing on the hypothesis put forward by Thomas (1947) to account for diurnal fluctuations in acidity 398
  A. Dark acidification and dark deacidification 398
  B. Respiratory quotients during dark acidification and dark deacidification . 398
  C. The inverse relation between CO_2 output and the rate of dark acidification as interpretable by the occurrence of a Wood & Werkman reaction . . 403
  D. The simultaneous absorption of carbon dioxide and oxygen by darkened shoots of *Opuntia* (the de Saussure effect) 405
III. Light deacidification, with incidental reference to dark deacidification and light acidification 407
  A. Light deacidification as resulting from the consumption in photosynthesis of respiratory and other carbon dioxide 407
  B. Values of assimilatory quotients during light deacidification in air as providing evidence that this process, like dark deacidification, is accompanied by intracellular CO_2 production 408
  C. The retardation of light deacidification and occasional induction of light acidification by CO_2 feeding 411
  D. The influence of CO_2 feeding on acid consumption in the dark and therefore on acid consumption in the light 414
  E. Volume changes in enclosed spaces and assimilatory quotients when leaves showing Crassulacean acid metabolism are illuminated in CO_2-enriched air 415
General conclusions 416

INTRODUCTION

The discovery by Wood & Werkman (see Werkman & Wood, 1942) that carbon dioxide is metabolically fixed in the synthesis of dicarboxylic acids by certain bacteria quickly led to the opening of a new field of experimentation in the realm of animal metabolism. Evidence was also soon obtained that CO_2 fixation accompanies acid production in yeasts and mould fungi. In 1945 the present writer failed to find any published records of experiments designed to determine whether a similar fixation occurs in green plants such as genera of the Crassulaceae and the Cactaceae, in which during certain seasons of the year there reside considerable powers of accumulating acids in the dark. Nor did he find any evidence that the abundant experimental results of assessable accuracy contained in the classical papers were being viewed from the new angle of the possibility that CO_2 fixation participates in the synthesis of vegetable acids from carbohydrates.

A fresh study of the informative articles in which Bennet-Clark (1933*a*, *b* and *c*) reported his own accurate experimental work and the views which he then held, and discussed the results obtained and conclusions drawn by some of his contemporaries and of the earlier pioneer investigators, soon showed that, if it were assumed that a Wood & Werkman reaction is an essential phase in acid synthesis by green plants, certain perplexing phenomena could be readily explained for the first time, and for others new explanations could be put forward, more convincing to the present writer than those which had been generally accepted by physiologists for seventy or more years.

This present article is, in part, an extended statement of one made verbally at a meeting of the Society for Experimental Biology in January 1947 and briefly in writing (Thomas, 1947) later in the same year. It will serve to introduce and to shorten reports on experiments performed at Newcastle in 1946, and subsequently, which were expressly designed to obtain direct or indirect evidence that carbon dioxide is an essential metabolite in the production of titratable acids, and that, because of this, what Bennet-Clark has called the Crassulacean type of acid metabolism may be attributed to changes in the internal concentration of carbon dioxide over a 24 hr. period under natural conditions. Further, some of the results of experiments carried out by earlier investigators of this type of metabolism will be considered, but, in order to save space, few of the conclusions will be discussed. The historian might maintain that many of the conclusions were reasonable at the time they were drawn, but the recorded facts now appear to find a better explanation in the light of present-day knowledge of the biochemistry of acid metabolism. Some of the older experiments were designed to get supporting evidence for conclusions directly opposite to those put forward in the present paper. That results obtained by such experiments can be accommodated within the new framework suggests that this is sound and testifies to the reliability of the earlier experimentalists.

Consideration of the results obtained in earlier work done elsewhere and in recent work at Newcastle places the Wood & Werkman reaction in the forefront of the picture of acid production. Giving this prominence to the reaction is the major contribution made in the present paper to theoretical knowledge concerning the causes of the diurnal changes in acidity that characterize Crassulacean acid metabolism. To elucidate the causes fully it is necessary, however, to study not only the conditions which promote acidification, but also those which influence deacidification. An attempt should be made to view acid metabolism as a whole. From his consideration of recorded facts and unpublished Newcastle data the present writer has concluded that carbon dioxide is liberated in the consumption of vegetable acids. This is a conclusion contrary to that drawn by some earlier authors. Accordingly, in certain directions the analysis of total acid metabolism will proceed here along new lines. In the main, however, earlier modes of analysis, sometimes slightly modified, and long-accepted descriptive terms, have been adopted. One of the objects of the present paper is to attach to a considerable body of empirical and theoretical knowledge acquired in the past, the reports to be made soon about experimental work performed at Newcastle.

I. ACIDIFICATION AND DEACIDIFICATION IN CRASSULACEAN ACID METABOLISM

A. *Peculiarities of Crassulacean acid metabolism*

From late spring to early autumn the titratable acidity of shoots or leaves of certain families (e.g. Crassulaceae, Cactaceae) may increase during the night, or when they are placed in the dark (see Bennet-Clark, 1933*a*). For many years the term *dark acidification* has been used to describe this increase in acid content. The amount of this increase may differ considerably from the amount of acid produced (see below). Under experimentally imposed conditions, acid accumulation may continue for longer than a summer's night. Malic acid is often predominant among the acids which accumulate; but not inconsiderable amounts of other acids, for example, citric and isocitric acids in *Bryophyllum* (Pucher, Leavenworth, Ginter & Vickery, 1947*b*, *c*), may be found at the end of a dark period.

With the return of daylight, or when leaves that have been kept in the dark for a period are illuminated, the amount of accumulated titratable acid decreases. The synonymous terms *light deacidification* and *photodeacidification* have been used to describe this decrease in acid content. Recent work (Pucher *et al.* 1947*a*, *b*, *c*) suggests that most or even all of the acids which accumulated during dark may be consumed in relative amounts roughly proportional to their concentrations at the moment of illumination. This means that if malic acid is predominant among the acids which accumulate, decrease in titratable acidity will be largely attributable to its consumption. This decrease may occur sharply. Titratable acidity often reaches a minimum value in the late afternoon or early evening. This minimum sometimes approaches zero; but, not infrequently, it is of considerable magnitude. The essential feature of Crassulacean acid metabolism is this diurnal fluctuation in titratable acidity. The main external governing factor is light intensity. Fluctuations similar to those occurring during day and night may be induced at will by the artificial illumination and darkening of leaves surrounded by ordinary air at a constant temperature.

In leaves kept for prolonged periods under experimental conditions in the dark the accumulation of titratable acid eventually stops. For a given leaf at a given time the value of the maximum reached is largely determined by the conditions which prevailed during the previous period of illumination (see below). If leaves are retained in the dark after the accumulation of titratable acid stops, a gradual decrease in acidity sets in. The leaves are then said to be undergoing *dark deacidification*.

B. *Acidification and deacidification as determined by the relative rates of the production and consumption of acids*

As defined above the terms acidification and deacidification refer to increases and decreases respectively in the amounts of accumulated titratable acid in leaves, and not to the metabolic processes by which acids are produced or consumed. These definitions are retained in this transitional stage in the study of acid metabolism, because they have been accepted for a long time. It is obvious that acidification is clear proof of the production of acid and deacidification of its consumption. But it is theoretically possible that acid may also be consumed during acidification and produced during deacidification, i.e. there may be, as de Vries (1884) postulated, a continuous metabolic production and consumption of acid. In general those who have worked since on acid metabolism have agreed with de Vries. On this view, acidification means that the rate of production is greater than that of consumption, and deacidification that acid is consumed faster than it is produced. Bennet-Clark has stated one of the important consequences: 'It is almost certain that formation and disappearance of acid both proceed continuously, and therefore the gross rates of acid formation or disappearance must exceed the net rates.' Introductory remarks are made on p. 411 of the present paper about the approximate evaluation of gross rates to be made by Thomas & Beevers (1949).

The assumption is made that in cells there are present enzyme systems, concerned with the production and consumption of acids, which are invariable and remain potentially active during alternating periods of day and night, under natural conditions, or of light and darkness under experimental conditions. Some of the crucial questions for which an answer has been sought at Newcastle are these: why do the relative rates of the reactions controlled by invariable enzymes concerned with the production and consumption of acids change (*a*) when leaves showing Crassulacean acid metabolism are subjected alternately to light and darkness, with resulting light deacidification and dark acidification respectively, and (*b*) after such leaves have been kept for prolonged periods in the dark, with the resulting substitution of dark deacidification for dark acidification?

The possibility is admitted that the *actual* activity of the invariable enzymes may be altered because of some quantitative or qualitative change (e.g. shift of pH, appearance or removal of an inhibitor), induced by the conditions mentioned in (*a*) and (*b*) above in their intracellular environment. As no evidence is known of such an alteration another possibility must be considered. It is possible that relative rates at any time will be determined by the active masses at enzymic centres of acid production and consumption in cells of those substrates which are concerned with these processes. In the next subsection reasons will be given for regarding a product of glycolysis and carbon dioxide as the substrates concerned with the production, and in the following one some conjectures will be made about the mechanism by which titratable acid is removed from cells.

C. *Acid production as resulting from glycolysis and* CO_2 *fixation*

The rate of formation of malic acid and the maximum amount which accumulates in Crassulacean leaves placed in the dark relates to the intensity of photosynthesis during the previous day. For this, and for other reasons (see Bennet-Clark, 1933*a*), it is now generally agreed that malic acid is derived from carbohydrate. Bennet-Clark stressed the point that the rate of glycolysis is one of the magnitudes which determines the rate of production of acids, and therefore of their accumulation. In his review he discussed several possible biochemical schemes which might account for the conversion of products of glycolysis into dicarboxylic and tricarboxylic acids. The arguments in the present paper, however, are based on another scheme which has been developed in the light of knowledge of facts about the heterotrophic assimilation of carbon dioxide that have been discovered since Bennet-Clark's review was written. These facts and the hypotheses relating to them have been summarized and discussed in authoritative articles written by Werkman & Wood (1942) and by Krebs (1943*a*).

The surprising results obtained by Wood & Werkman in their researches of 1936 and 1938 on the production of succinic acid by propionic bacteria opened a new chapter in the study of acid metabolism. They discovered that little or no succinic acid accumulated in the absence of carbon dioxide, and that in the presence of this gas 'there was a net decrease in CO_2 of the system and in amounts equivalent to the succinic acid formed'. It has since been established that carbon dioxide is assimilated in the metabolic production of acids by other bacteria and by certain mould fungi and animal tissues.

Although it had been suggested (see, for example, Barron, 1943) that the fixation reaction of Wood & Werkman may play a part in the formation of vegetable acids by green plants, as far as the present writer knows, when Thomas & Beevers began their investigations on *Bryophyllum*, the only evidence relating an absorption of carbon dioxide to the production of carboxylic acids by green plants was that which had been obtained incidentally to the experimental study of the dark reaction in photosynthesis. Upon transfer from the light to the dark, certain green plants can 'pick-up' carbon dioxide for a short period. Work with labelled carbon atoms indicates that some of the carbon contained in the absorbed gas becomes incorporated in carboxylic groups (see Rabinowitch, 1945); but it has not yet been firmly established whether or not this carbon dioxide pick-up is attributable to a fixation reaction of the Wood & Werkman type. The possibility, which may seem rather remote at present, is noted that Crassulacean acid metabolism may be a phenomenon which occurs in many green cells, but is augmented to easily measured dimensions in Crassulacean leaves and shoots of succulent plants. At this stage, however, the production and consumption of vegetable acids in relation to possible intermediate stages in photosynthesis and aerobic respiration will not be considered.

Nor will more than superficial consideration be given to biochemical details concerning the reactions by which titratable acids are produced from non-acidic substances, and consumed so as to bring about a loss in titratable acidity. Wood & Werkman suggested that either pyruvic acid or phosphopyruvic acid, both of which are products of glycolysis, is the actual compound that combines with carbon dioxide, and that oxalacetic acid is the first dicarboxylic acid to be produced. An enzymic study by Krampitz & Werkman (1941) with a thermolabile preparation from *Micrococcus lysodeikticus* provided evidence for the occurrence of the reversible reaction

$$CO_2 + CH_3.CO.COOH \rightleftharpoons COOH.CH_2.CO.COOH.$$

More recently, Gollub & Vennesland (1947) have reported the presence in parsley root of an oxalacetate carboxylase that, in a system of some complexity, can promote the fixation of carbon dioxide in the synthesis of oxalacetic acid as well as actuate the decarboxylation of this acid. It may well be that the equation written above is a simplified representation of an enzymic reaction which will only continue when linked with other reactions.

Krebs (1943*b*) has discussed his and other schemes which have been constructed to account for the production from oxalacetic acid of malic acid and other C_4 dicarboxylic acids, and of di- and tricarboxylic acids containing more than four carbon atoms. In this present simplified introduction to what may well prove to be a complicated story, titratable acid will be regarded as composed exclusively of malic acid. As has been stated above, other acids may be produced and consumed with malic acid. In *Bryophyllum*, for example, isocitric acid, citric acid, and traces of succinic and oxalic acid are among the other acids which may be present (see Pucher *et al.* 1947*a*, *b* and *c*). A fixation of carbon dioxide independently of that occurring in the synthesis of oxalacetate may take place in the formation of some of these other titratable acids. Ochoa (1945) has already reported the presence in pig heart of an oxalosuccinic carboxylase which can promote the combination of carbon dioxide and α-ketoglutaric acid as well as actuate the decarboxylation of the synthesized product, oxalosuccinic acid. The equation written below represents the reversible reaction:

$$CO_2 + COOH.CO.CH_2.CH_2.COOH \rightleftharpoons COOH.CO.CH(COOH).CH_2.COOH.$$

The equation indicates that in the fixation reaction a third titratable hydrogen ion is formed. Ochoa gives evidence that the action of oxalosuccinic carboxylase (which requires manganese ions) is linked with that of isocitric dehydrogenase (involving the presence of triphosphopyridine nucleotide) and that of aconitase. The oxidation of glucose-6-phosphate to 6-phosphogluconate may play a part in the change of oxidized T.P.N. to reduced T.P.N.

At Newcastle, for the present, we are not taking into account complexities resulting from possible linkages with other metabolic systems of those cell mechanisms which are concerned with the synthesis of carboxylic groups. Nor are we considering stages in acid metabolism, although a knowledge of them is admittedly essential for the internal control of gaseous exchanges to be fully understood. The resulting increases in titratable acid, and gaseous exchanges accompanying them, have alone been measured. The overall equation (*b*) on p. 398, has been used to epitomize the contention that, in the formation of malic acid from a product of glycolysis, carbon dioxide is metabolically fixed. Experiments are in progress to determine to what extent oxygen supply may influence the rates of the production and consumption of acids. We have in mind the possibility that, whereas oxygen may not directly influence the production of acid, it may, by regenerating hydrogen acceptors in aerobic glycolysis, control the continued production of acid from carbohydrate.

D. *The reduction in titratable acidity through the consumption of vegetable acids*

The equations written in the previous subsection imply that decarboxylations of keto acids lead to a decrease in titratable acidity. Since cell mechanisms are known by which malic acid and other acids containing alcoholic groupings are oxidized to keto acids, it is a reasonable hypothesis that carboxylase action may play at least a major part in the consumption of vegetable acids. Light and dark deacidification, which occur when the rate of consumption is greater than that of production, would then be accompanied by a CO_2 output. This point will be taken up below.

Whereas there is little doubt that carbohydrate is the food substrate from which vegetable acids are derived, it is still undecided what are the metabolic fates of substances formed by the decarboxylation of di- and tricarboxylic acids. The two possibilities to be borne in mind are that these substances may be aerobically oxidized according to the overall equation

$$C_4H_6O_5 + 3O_2 = 4CO_2 + 3H_2O,$$

or, as Bennet-Clark suggested, they may undergo oxidative anabolism and be reconverted into carbohydrate. Thomas (1947, p. 327) has hinted that a possible overall equation to represent this reconversion may be written thus:

$$2C_4H_6O_5 = C_6H_{12}O_6 + 2CO_2.$$

E. *Previous hypotheses to account for diurnal variations in titratable acidity, with special reference to Wolf's hypothesis*

Since Crassulacean acid metabolism is shown only by plant organs which contain chloroplasts (see, for example, Warburg, 1886), light energy absorbed by chloroplast pigments plays either a direct or an indirect part in the process of light deacidification. Bennet-Clark has described and discussed most of the hypotheses put forward prior to Wood & Werkman's discovery that carbon dioxide is fixed in acid synthesis. The only ones that concern us are those that assign the control of the relative rates of acid production and consumption to variations in the composition of the internal atmosphere.

Astruč (1903) stated clearly the salient points, which, indeed, were well understood by earlier workers such as Mayer (1878), Kraus (1883), de Vries (1884), Warburg (1886), and Aubert (1892). In all green parts of plants, when the rate of photosynthesis exceeds that of respiration in the light, the internal atmosphere tends to become enriched in oxygen and impoverished in carbon dioxide. In the dark, or in the light when photosynthesis is feeble and respiration vigorous, the internal concentration of carbon dioxide tends to increase and that of oxygen to get less. In such plants as are succulent (for example, the Cacti) among those which show Crassulacean acid metabolism these tendencies will be accentuated, and in them differences between the composition of the internal and external atmospheres will be greater than in normal plants. Although some of the plants (e.g. *Bryophyllum*) showing diurnal effects are not succulent, and although experimental results (see Warburg, 1886; Astruč, 1903) point to the promotion rather than to the retardation of acid accumulation when the external atmosphere is enriched with additional oxygen, Pfeffer and other leading physiologists have maintained that acid accumulation in the dark results from the incomplete oxidation of sugars owing to a shortage of oxygen in the internal atmospheres. For example, Richards (1915) held that acid production results from a comparative lack of oxygen in the massive tissues of succulents. More recently Gustafson (1925), in discussing the results of his well-controlled experiments, wrote: 'The formation of acid is a normal phenomenon in *Bryophyllum calycinum* and is undoubtedly due to incomplete respiration, brought about by lack of oxygen and perhaps the slow escape of CO_2.' He gave reasons for concluding that gaseous exchanges between *Bryophyllum* leaves and the outside atmosphere may be slow although the leaves are not succulent. It is of interest that Gustafson thought that acidification might possibly be controlled in some way by carbon dioxide. As will shortly be apparent, the present writer would modify Gustafson's conclusion and attribute the slow escape of CO_2 to the formation of acid.

It has not always been clearly stated that there is a twofold relation between oxygen concentration and the acid accumulation which results because the rate of acid production exceeds that of consumption. If importance attaches to oxygen concentration as a controlling factor in acidification and deacidification, consideration must be given to the influence of oxygen on the cellular consumption of acid as well as on its production. Astruč has laid sound foundations for future researches on this aspect of acid metabolism. It remains to be determined whether or not acid consumption is augmented by the enrichment in oxygen of the internal atmosphere owing to photosynthesis and retarded by the fall in the internal oxygen concentration when leaves are respiring in the dark. Bennet-Clark has, however, marshalled the evidence that points decisively against the long-held view that when acid accumulates in the dark it does so because there is a shortage of oxygen in the internal atmosphere. It still remains possible, however, that, when the oxygen supply is subnormal, the dark accumulation may be greater under certain conditions owing to retarded consumption, and that light deacidification may proceed faster in supernormal oxygen concentrations. These points of detail are under investigation at Newcastle.

The main conclusion stated by Bennet-Clark seems, however, to be well established. Diurnal fluctuations in acidity are not attributable to such fluctuations in the internal oxygen concentration as occur even in the most succulent of the plants that show Crassulacean acid metabolism.

The other variable, relating to alternations of light and darkness, is the internal concentration of carbon dioxide. At Leipzig, Ruhland & Wetzel (1931) and Wolf (1931) attached crucial significance to diurnal variations in this concentration. They pointed out that light deacidification occurs when illuminated green cells consume carbon dioxide in photosynthesis, and asserted that, as night follows dusk, respiratory CO_2 will tend to accumulate in the cell sap and intracellular spaces and eventually occasion qualitative changes in carbohydrate and acid metabolism. On inadequate evidence they suggested that pyruvic acid, a product of glycolysis, might have different metabolic fates in the light and in the dark, owing to inhibitory effects on carboxylase indirectly induced by the respiratory CO_2 which accumulated in the dark. They thought it was possible that the phenomenon described as CO_2 zymasis by Thomas (1925) might occur under natural conditions in the dark. This process may indeed lead to the accumulation in apple fruits, and in certain other organs, of acetaldehyde in concentrations that may considerably reduce carboxylase activity. The Leipzig workers postulated that such concentrations were reached in darkened leaves showing Crassulacean acid metabolism. The present writer's findings (see Thomas, 1947, p. 327) agree with Bennet-Clark's (1933*b*) that very little acetaldehyde is ever present in such leaves under natural conditions. Moreover, in the light of the results of much unpublished work on CO_2 zymasis he can assert with some confidence that this form of disordered carbohydrate katabolism is not induced, even in organs such as apple fruits, which are relatively susceptible to it, until the *external* CO_2 concentration exceeds 40%. The internal concentration will certainly be markedly higher. Under natural conditions cells would probably be under anaerobic conditions if the internal CO_2 concentration rose much above 20%. For healthy leaves living in air there is no published evidence of values approaching such concentrations.

The Leipzig views on the biochemistry of acid production will not be discussed further; but consideration will be given on p. 415 to some of Wolf's conclusions about acid consumption, based on the results of his experiments on light and dark deacidification, which he believed, for good reasons, were controlled by the same metabolic system. The positive contribution made by the Leipzig workers was to draw attention to the possible cardinal importance (cf. Gustafson's qualified statement) of carbon dioxide rather than of oxygen in controlling diurnal fluctuations in the acidity of the green cells of plants showing Crassulacean acid metabolism. The present writer felt that this possibility became a probability when he had considered the experimental results obtained at Leipzig and elsewhere in the light of Wood & Werkman's discovery.

II. EVIDENCE BEARING ON THE HYPOTHESIS PUT FORWARD BY THOMAS (1947) TO ACCOUNT FOR DIURNAL FLUCTUATIONS IN ACIDITY

A. *Dark acidification and dark deacidification*

A number of peculiar facts about the gaseous exchanges that may accompany dark acidification in the Crassulacean type of acid metabolism have been reported and variously explained during the last century and a half. In the late summer of 1945 the present writer came to the conclusion that metabolic fixation of carbon dioxide by the Wood & Werkman reaction would afford a better explanation of these facts than any previously put forward. He postulated that when such plants were placed in the dark after a period of illumination there would occur simultaneously at least two metabolic processes which affect the composition of their external atmosphere. In the normal aerobic respiration of carbohydrate, oxygen would be absorbed from the air and carbon dioxide would be produced by the respiring cells. If all this carbon dioxide escaped the value of the R.Q. would be unity. The overall equation for sugar respiration written in its old form is

$$C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O. \qquad (a)$$

In quantitative experiments on respiration one measures the apparent oxygen uptake and CO_2 output. The idea that these magnitudes are not necessarily the same as real oxygen consumption and CO_2 production by living cells is familiar enough because of our knowledge of respiration in dimly illuminated green cells. Until, however, the possibility was considered that a Wood & Werkman reaction may take place in green plants there was no reason to suppose that internal events, apart from retardations resulting from increases in resistance to diffusion, might cause CO_2 output in the dark by such plants to differ from CO_2 production. If, however, to quote Thomas (1947, p. 328), 'the stages in the production of malic acid from hexose are (*a*) glycolysis with the formation of pyruvic acid (or of a phosphorylated variety), (*b*) condensation of pyruvic acid with carbon dioxide produced by cell respiration, yielding oxalacetic acid, and (*c*) reduction of oxalacetic acid to malic acid', it is clear that, owing to reaction (*b*), CO_2 output would be less than CO_2 production by the amount of carbon dioxide metabolically consumed in acid synthesis. Thomas summarized the partial reactions symbolically thus:

$$C_6H_{12}O_6 \xrightarrow{-4H} 2C_3H_4O_3 \xrightarrow{+2CO_2} 2C_4H_4O_5 \xrightarrow{+4H} 2C_4H_6O_5.$$

He stated that the partial equations 'satisfy the arithmetical demands of the overall equation'

$$C_6H_{12}O_6 + 2CO_2 = 2C_4H_6O_5. \qquad (b)$$

Equations (*a*) and (*b*) will play an important part in the analysis of results reported in the opening papers of this series.

B. *Respiratory quotients during dark acidification and dark deacidification*

Nearly 150 years ago de Saussure (1804) made a striking discovery about the gaseous exchanges in the dark between green parts of cacti and other succulent plants and their surrounding atmosphere. Pointing out the striking analogy with the respiratory activity of animals, but disregarding the means by which gaseous exchange is effected, he used the terms inspiration and expiration to describe the intake and output of gases by plants.

Authors (e.g. Warburg, 1886) continued to use these terms later in the century. De Saussure found that whereas most forms of vegetation, gathered after a genial summer's day and placed in an enclosed space in the dark, remove oxygen from and liberate carbon dioxide into the atmosphere surrounding them, green parts of succulent plants may behave differently. He reported that, under certain conditions, *Les feuilles ou les parties vertes du cactus opuntia, de la crassula cotyledon, du sempervivum tectorum, de l'agavé Americana, de la stapelia variegata, diminuent le volume de leur atmosphère en absorbant du gaz oxygène, sans qu'il se forme sensiblement de gaz acide carbonique.* In his famous experiment on the inspiration of *Opuntia*, he placed 119 c.c. of branches or leaves in a receptacle, sealed by mercury, which contained 951 c.c. of air deprived of carbon dioxide. He selected these relative volumes to ensure that volume changes would be perceptible after a night in the dark, and that there would be sufficient oxygen throughout to maintain health in the parts of the shoots used. The next morning at sunrise, after correcting for changes in temperature and pressure, de Saussure found that the volume of the atmosphere around the shoots had diminished by 79 c.c. (i.e. they had inspired three-quarters of their own volume of gas), and that 79 c.c. of oxygen had been removed from the atmosphere. The clearness at the end of the experiment of the lime water he had placed inside the receptacle led him to conclude that in the residual air there did not remain *un atôme de gaz acide carbonique.* Behind de Saussure's attempts to explain the experimental facts he discovered there appears to have been an idea that inside the cactus oxygen is converted into carbon dioxide, which is in some way retained by the plant.

De Saussure realized that this unusual form of gaseous exchange was peculiar to succulent and certain other plants, and it was discovered later that it is related to the accumulation of acids. During, and only during, the accumulation of acids, the R.Q. of all parts of plants showing Crassulacean acid metabolism is always less than unity and may actually be zero.

Until the Wood & Werkman reaction was discovered, low values for R.Q.'s were attributed to incomplete oxidation of carbohydrate, involving oxygen uptake. For example, Bennet-Clark (1933*b*) stated: 'Whilst malic acid is accumulating, the R.Q. has a very low value (often as low as 0·1), since oxygen is consumed in the conversion of sugars to malic acid without any corresponding output of CO_2.' Physiologists appeared to find this a reasonable explanation, ignoring the implication that normal aerobic respiration of sugars must cease when the value of the R.Q. turned out to be zero.

In the light of the Wood & Werkman reaction, Thomas (1947, p. 268) explained the inner causes of these low R.Q.'s in a new way. Referring to a value of 0·03 for darkened shoots of *Opuntia*, recorded in Table XII of his book, he wrote: 'Conceivably the normal respiratory oxidation of hexose and the synthesis of dicarboxylic acids and their derivatives are occurring as collateral processes. Some, or even all of the CO_2 *produced* in the reaction above by the overall equation (*a*) may be consumed as a metabolite in the production of acids according to the overall equation

$$C_6H_{12}O_6 + 2CO_2 = 2C_4H_6O_5.$$

If all were consumed the CO_2 *output* by the whole shoot would be zero, and, since the oxygen uptake is positive, the R.Q. would be zero. In the experiment which gave the result recorded in Table XII, a small fraction of the respiratory CO_2 escaped and, accordingly, a positive but very low R.Q. was obtained....Evidently, according to the present writer's interpretation, the value of the apparent R.Q. will approach closer to unity as the rate of CO_2 production in real respiration increases relatively to the rate of CO_2 consumption in the synthesis of acid.'

There are many records of respiratory quotients ranging from zero to unity for tissues undergoing dark acidification. Aubert (1892) allowed plants to respire over dark periods of from 12½ to 18 hr., and obtained, for the periods, averages which ranged from 0·60 for *Opuntia tomentosa* and *Mamillaria* sp. to 0·88 for *Sedum reflexum*. Even when the CO_2 outputs were low, the oxygen uptakes were not inconsiderable. From some of the experimental results recorded in the older literature it has been concluded that there exists, in general, an inverse relation between the magnitudes of acidification and of the R.Q. For example, Astruč (1903) obtained a value of about 0·5 for the R.Q. of the young central leaves of *S. tectorum*, in which there was less than a 30% increase in titratable acidity during the period of exposure to the dark, while over the same period the acidity of adult peripheral leaves of the rosette increased over 200% and had R.Q.'s ranging down from 0·16 to 0·08. The youngest leaf of a plant of *Echeveria glauca*, showing virtually no dark acidification, had a R.Q. of 0·98; while, in the oldest leaf the malic acid content increased from 1·2 to 2·9 mg./g. fresh weight and the R.Q. was 0·40. Astruč also found that under conditions (e.g. a relatively high temperature) that caused a diminution of dark acidification, the value of the R.Q. was higher than that of comparable leaves undergoing more vigorous acidification. The instances could be multiplied from the records of experiments at different temperatures reported by Warburg (1886) and others. Astruč's general conclusion was *plus le quotient respiratoire s'écarte audessous de l'unité, plus il se forme d'acide organique*. The present writer would now prefer to state, the greater the amount of acid accumulation the more the R.Q. will fall below unity; for, according to his hypothesis, if no acid is synthesized all the carbon dioxide produced in real respiration would escape and the respiratory quotient would be unity; but, if acid is synthesized, respiratory carbon dioxide becomes fixed in a Wood & Werkman reaction commensurately with the extent of acidification (equation (*b*) above), and only a fraction, or even none at all, escapes and changes the composition of the outside atmosphere. Inevitably, the result will be a fall in the measured R.Q. to an extent which is inversely related to the net amount of carbon dioxide fixed during acid accumulation. On this view, put forward in the light of modern knowledge, Astruč's finding that the difference between CO_2 production and CO_2 output was less, and in consequence that the R.Q.'s were higher, in the younger leaves than in the older leaves of *Sedum tectorum* and *Echeveria glauca*, may possibly be attributed to the greater powers possessed by the older leaves of fixing carbon dioxide and, therefore, of synthesizing acid. When § III of the present paper has been read, it will be obvious that statements must be guarded, since Astruč gave no data from which the relative powers of acid consumption possessed by younger and older leaves, might be assessed.

Under all conditions in the dark, whether acidification is occurring vigorously or not at all, oxygen uptake continues (see, for example, the statement made above about Aubert's experiment). It is a significant fact that no one has succeeded in relating the net amount of oxygen consumed to the amount of acid accumulating in *Opuntia* or other genera for which R.Q.'s approaching zero have been found. Tentatively the present writer will assume that oxygen uptake is exclusively confined to the normal aerobic respiration of carbohydrate, which goes on continuously in all plants showing the Crassulacean type of metabolism. It is admitted that, as knowledge grows, this assumption may require some modification. The results of experiments recorded in the older literature and of those recently performed at Newcastle strongly suggest, however, that the major fraction, at least, of the oxygen absorbed from the atmosphere is consumed according to the overall equation (*a*) written on p. 398. Further discussion will be postponed until the Newcastle work is reported.

A point that will be emphasized in later papers is that, in their evaluations of R.Q.'s, most of the pioneer investigators allowed green shoots to remain in the dark for periods of from 8 to 24 hr., or even longer, before measuring changes in the composition of the external atmosphere. Their results (see, for example, Aubert's recorded above) are often averages for rather prolonged periods. By our experiments at Newcastle we have proved that during the opening hours of dark acidification the values of R.Q.'s change whilst the rate of this process rises and falls. Later discussions will not now be anticipated further than to state that an evaluated R.Q., such as Aubert's of 0·88 for *Sedum reflexum*, may have been an average of values near zero at the outset of the dark period, while the fixation of respiratory CO_2 was vigorous, and of progressively rising values which approached unity towards the end of the period of acid accumulation.

Aubert realized that R.Q.'s may rise over a period which terminates in the replacement of dark acidification by dark deacidification. For example, he reported that for one night in the dark the R.Q. of leaves of *Crassula arborescens* was 0·24, but that it approached unity during a subsequent dark period. These were average values. The second one might well have meant that the final R.Q. was well above unity (see below).

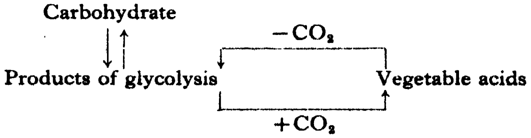
When acid accumulation has reached its maximum value the rate of acid consumption will, of course, be equal to that of acid production. If at this stage it can be shown that the value of the R.Q. is maintained at unity two conclusions may be drawn: first, that in the production and consumption of titratable acid the net turn-over of carbon dioxide (and of oxygen, if it plays a part) is zero when the rates of production and consumption of acid are equal; and secondly, that normal aerobic respiration of carbohydrate is the only other metabolic process involving a net oxygen uptake and CO_2 output. The first conclusion would bear on the elucidation of certain problems discussed in § III on the consumption of acids. If we concede that 1 g.mol. of carbon dioxide is metabolically fixed for every g.mol. of malic acid synthesized (equation (*b*) on p. 398), we should also have to allow that 1 g.mol. of carbon dioxide is liberated for every g.mol. of malic acid consumed. (In parentheses we may note that equation (*c*) on p. 409 would satisfy this requirement.) Possibly the only other way to account for an R.Q. of unity at the stage of maximum acid accumulation would be to set aside the hypothesis that production and consumption of acids go on continuously, and to postulate either that they both stop at this stage and at this stage only, or that prior to this stage production alone goes on and after it consumption alone. It may be predicted that no one would consider either of these postulates seriously.

After long periods in the dark, deacidification replaces acidification, possibly owing to such a decline in the rate of glycolysis that, even with an ample supply of carbon dioxide, the rate of acid production becomes less than that of acid consumption. Bennet-Clark (1933*b*) attached importance and consequence to the fact that R.Q.'s rise above unity, and at times much above unity, while dark deacidification is in progress. Citing Astruč's experimental results, he wrote: 'in many cases the values recorded by him are between 1·00 and 1·33, but occasionally higher values were recorded. The following values are taken from his paper: 1·50, 1·48, 1·53, 1·75, 1·78, 2·41, 1·42. Astruč makes no comment on their significance.'

He referred to his own work (1932) in which 'continuous records were obtained of the R.Q. by a method in which the leaves are kept under constant conditions of temperature and external CO_2 tension. High initial values of the R.Q. were found: during the phase of acid accumulation values much lower than unity were obtained, and these were succeeded by values higher than 2·0 during the phase of acid disappearance. Since the plants had been in equilibrium with their environment from the start of the experiment, the high values of the R.Q. obtained at the end are clearly not due to CO_2 coming out of solution.'

Bennet-Clark realized, of course, that if malic acid is completely oxidized (equation (*d*) on p. 409 of the present paper), the R.Q. would be 1·33, but lower if malic acid and sugar are simultaneously oxidized. He then pointed out that 'the high values found by Astruč could be explained either by the partial oxidation of the malic acid, or by the conversion of part or all of the malic acid carbon into carbohydrate and the storage of this carbohydrate in the tissues'. The trend of his discussion of his own experimental results and of those of others shows his strong inclination to favour the view that, in disappearing under certain conditions, all the malic acid may be converted into carbohydrate by oxidative anabolism. A major problem is the elucidation of the mechanism of such a form of oxidative anabolism. It may possibly be solved by the application of recently developed ideas about the cycle of changes in the metabolism of di- and tricarboxylic acids and about the reversibility of glycolysis.

When he formulated his hypothesis Bennet-Clark thought that all the acid is converted into compounds other than carbon dioxide. Since his arguments (see, for example, p. 403) would probably have taken a different turn had he known about the Wood & Werkman reaction, they will not be discussed here. The present writer thinks that carbon dioxide would be a product of the reaction by which malic acid is continuously converted into carbohydrate in the light as well as in the dark. Statements bearing on this view are made on pp. 401 and 409 of the present paper. An outline scheme of the interconversion of carbohydrate and vegetable acids is given below:



It is assumed that, before profound starvation sets in, oxygen uptake is exclusively attributable to the normal aerobic oxidation of carbohydrate, and that it continues throughout the phases of dark acidification and dark deacidification. The scheme will account for the facts that (*a*) when acidification is occurring there is a net consumption of respiratory CO_2, with a resulting R.Q. less than unity, and (*b*) when deacidification takes place there is a net liberation of carbon dioxide which supplements respiratory CO_2, and causes the R.Q. to rise above unity. The point is again made that the demonstration of a R.Q. of unity when acid accumulation is at its maximum would provide solid evidence in favour of the view that the same volume of carbon dioxide is liberated in the consumption of one molecule of malic acid as the potential volume of respiratory carbon dioxide which is metabolically consumed in the synthesis of one molecule of this acid.

C. *The inverse relation between* CO_2 *output and the rate of dark acidification as interpretable by the occurrence of a Wood & Werkman reaction*

In experiments designed to determine whether regular quantitative relations exist between the progressive increase or decrease in acid accumulation and the rate of CO_2 output in darkened green parts of plants showing the Crassulacean type of metabolism, Bennet-Clark (1930) made simultaneous measurements of their magnitudes over a period of 80 hr., using leaves of *Sedum praealtum* kept in continued darkness at 21° C. He later wrote (1933*b*) about this experiment: 'The rate of CO_2 output is high immediately after the leaves are placed in darkness (at 8 p.m. on 16 June), but very rapidly the rate falls... concurrently with a rapid increase in malic acid content.' The charts drawn by Bennet-Clark indicate that the minimum rate of CO_2 output was reached in rather less than 10 hr., and slightly before the amount of accumulation had progressed to its maximum value, when, of course, the rate of acid consumption would equal that of acid production. (We note in passing that if the arguments put forward in the previous subsection are valid, the R.Q. would then have risen to unity.) Subsequently the acid content fell sharply to reach a minimum value at about 30 hr. from the beginning of the experiment. Clearly, during this phase dark deacidification occurred vigorously, i.e. the rate of acid consumption greatly exceeded that of acid production. Next there occurred a secondary rise in acidity (i.e. a second phase of dark acidification), giving a peak value after a few hours. This was followed by a second phase of dark deacidification which continued for the remainder of the experiment. From its minimum value reached at about 10 hr. after the beginning of the experiment the CO_2 output rose during a period of several hours to reach a value that remained fairly steady until the second phase of dark acidification set in. The chart for CO_2 output drawn by Bennet-Clark shows a second trough which roughly coincides with the second peak in the chart for acid accumulation. Bennet-Clark concluded: 'These results indicate that malic acid is not the substrate from which carbon dioxide is formed.' In 1945 the present writer, knowing about the Wood & Werkman reaction, which had not been discovered when Bennet-Clark performed his pioneering experiments, considered that these results might have another meaning. They might indicate that carbon dioxide is one of the substrates in the reaction from which malic acid is formed.

In commenting on the fluctuations shown in his charts Bennet-Clark wrote: 'It is important to note that the maxima on the acidity-time curve do not coincide with maxima on the CO_2 output-time curve. They nearly, but not exactly, coincide with the minima on the CO_2 output-time curve. Similar results have been obtained in all experiments with both *Sedum praealtum* and *Crassula lactea*, the two species examined.' Thomas (1947, p. 327) suggested that 'one possible explanation of the troughs in the CO_2 output curve... is that they correspond to active periods of CO_2 assimilation in the production of acids in the dark'. The striking and, at the time it was discovered, surprising near coincidence of CO_2 troughs and acid peaks, so clearly shown by Bennet-Clark's graphical representation of his results and testifying to the accuracy of his work, may provide the first pictorial evidence that respiratory carbon dioxide produced by green plants instead of escaping, to be measured as CO_2 output, is metabolically consumed in the synthesis of acid. The present writer considers that where troughs occur in Bennet-Clark's charts for CO_2 output, the Wood & Werkman reaction was proceeding vigorously. In consequence the rate of acid production exceeded that of acid consumption. For total acid metabolism there was a net CO_2 absorption. Respiration was the only internal process which supplied carbon dioxide for consumption at the centres of acid synthesis. Accordingly, although respiratory carbon dioxide was produced with undiminished intensity, the CO_2 output fell by an amount equivalent to the carbon dioxide metabolically consumed. Obviously, on this interpretation, the R.Q. would be less than unity, for undiminished respiratory intensity implies unchanged oxygen uptake.

There is other evidence that can be placed in line with Bennet-Clark's. Thoday & Richards (1944) measured the rate of CO_2 output by leaves of *Kleinia radicans* during starvation in the dark. Their experiments also were so accurate that marked changes in rate during short intervals were clearly discernible. They recognized several phases in the graphs which recorded their July experiments. The initial phase, which was passed through immediately after the dark period began, is the one that interests us here. The rate of CO_2 output fell to a low level during the first 12 hr. (i.e. when acidification was probably occurring rapidly and a major fraction of respiratory CO_2 was fixed) and then rose rapidly to a peak during the next 12 hr. (presumably as the rate of acidification progressively declined, and a progressively greater fraction of respiratory CO_2 escaped). (We note, in parentheses, that with such sharp changes in CO_2 output, R.Q.'s may also alter continuously. An average R.Q. obtained over the opening period of 12 hr. would be very different from that obtained over a 24 hr. opening period (cf. remarks on p. 401).) Thoday & Richards emphasized the point that in the summer the darkened leaves for 1 or 2 days showed diurnal fluctuations in CO_2 output very similar to those found by Bennet-Clark for leaves of *Crassula lactea*, but that as the season advanced this initial phase disappeared. They state that older plants, placed under experimental conditions in October, give 'no evidence of the earlier part of the July graphs. The CO_2 output rises from the beginning and not until 60 hr. does it fall.' Now, it was established in the last century that many plants showing Crassulacean acid metabolism do so only over a limited period in the year. It is possible therefore that by October the leaves of *Kleinia radicans* had lost the power to undergo dark acidification. There would then be no net fixation of the carbon dioxide produced in respiration, and, consequently, no trough in the CO_2 output curve. The initial phase seen in the graphs for the July experiment would be absent. From September cuttings Thoday & Richards succeeded in raising plants which showed the initial phase in experiments performed late in the year. It is possible that the leaves of these youthful plants had the power of undergoing dark acidification. The experiments carried out at Bangor may therefore provide evidence that troughs in the CO_2 output curves of the kind we are considering only occur when the rate of acid production exceeds that of consumption, i.e. when a Wood & Werkman reaction is proceeding with relative vigour.

At Leipzig, Wolf (1931) argued that if malic acid is produced in the Crassulaceae by what is known as the Thunberg reaction (see Bennet-Clark, 1933*b*), for every molecule of acid synthesized two molecules of carbon dioxide must be liberated. He performed a critical experiment over a temperature range from 10 to 40° C. to test this argument, measuring simultaneous CO_2 output and acid accumulation at each selected temperature. Broadly, he found that over the lower temperature range (say 10–20° C.) dark acidification was relatively vigorous and CO_2 output was at a low level, whereas at the higher temperature the vigour of dark acidification declined while the level of CO_2 output rose.* After performing experiments to test it, he gave good reasons for discarding the idea that the results were explicable in terms of a greater solubility of carbon dioxide at the lower temperature, and he rightly concluded that the results of his experiment provided no evidence in support of the Thunberg hypothesis. He wrote: *Es ist vielmehr für unsere Befunde beizeichnend, dass gerade bei ausserordentlich reger Säurebildung nur minimale, kaum fassbare* CO_2*-mengen ausgeschieden wurden.* He laid emphasis on the tendency for an inverse proportionality to exist between the intensity of acid-building and of CO_2 escape. This tendency impressed other members of the Leipzig school. Ruhland & Wetzel (1931) referred to the experimental findings of Wolf *dass ein Maximum der Äpfelsäureanhäufung in den Crassulaceen stets von einem Minimum der* CO_2*-Ausscheidung begleitet ist.* This statement is still acceptable, but, on the basis of the assumption that a Wood & Werkman reaction participates in acid accumulation, exception may now be taken to the statement by Wetzel (1931), justifiable at the time he made it, *die Anhaüfung von Äpfelsäure stets von einer ausserordentlich starker Senkung der* CO_2*-Produktion begleitet ist.* The main theme of the present subsection has been that CO_2 production in respiration, or in other forms of metabolism, will be greater than CO_2 escape from green organs that are accumulating acids, because a fraction of the respiratory CO_2 is fixed by a Wood & Werkman reaction. Thomas (1947, p. 328) cited the Leipzig experiments as providing evidence in support of his hypothesis.

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* This effect of temperature on acidification was discovered by de Vries (1884). At Newcastle Dr S. L. Ranson has obtained evidence substantiating and extending Wolf's results. Further discussion of temperature effects is therefore postponed until these results are published.

Finally, it may be noted that, if the normal aerobic respiration of carbohydrate and acid metabolism are the only processes which affect CO_2 output in darkened leaves before starvation sets in, troughs in the CO_2 output curves for plants kept under constant external conditions and not showing stomatal movements should be found only when acidification is taking place; and the depths of the troughs should relate to the intensity of acidification. Published data are insufficient to substantiate these statements at present. These relations would, of course, account for the direct relations between the extent of dark acidification and the fall of R.Q.'s from unity towards zero, on which the pioneer workers on acid metabolism rightly laid so much stress.

D. *The simultaneous absorption of carbon dioxide and oxygen by darkened shoots of* Opuntia *(the de Saussure effect)*

Having demonstrated that, in the dark, the leaves or green parts of certain succulent plants, by absorbing oxygen from a CO_2-free atmosphere without liberating appreciable amounts of carbon dioxide, cause a diminution in the volume of the atmosphere in the vessel in which they were enclosed, de Saussure (1804) repeated the experiment, using segments of *Opuntia* in an atmosphere containing 74% nitrogen, 19% oxygen and 7% carbon dioxide. He observed that the diminution in volume, to which he referred as inspiration, occurred more quickly in the atmosphere enriched in carbon dioxide than in the absence of this gas. After 12 hr. in the dark the volume had diminished by 20%, corresponding to an inspiration by the leaves of gas up to about one-quarter of their volume. Analysis showed that the leaves had inspired 13·5% oxygen and 5·5% carbon dioxide. He stated: *Elles avaient donc inspiré les deux gaz en même raison, ou les ¾ de l'un et de l'autre. Après cette inspiration elles étaient saturées; elles n'ont pu continuer à diminuer leur atmosphère par une obscurité prolongée.* Over 140 years later we can account for these and other experimental results reported by de Saussure in terms of CO_2 fixation in acid synthesis simultaneously with oxygen absorption in normal aerobic respiration. The reduction in volume would stop when dark acidification reached its peak value.

It is not surprising that de Saussure's explanations of the results of his many ingenious experiments were not acceptable to Warburg (1886), who, however, found the facts de Saussure recorded to harmonize with the results he himself obtained in experiments on inspiration and expiration during acid metabolism. Nor is it surprising that Warburg's own explanations appear confused to us at the present day. They will therefore not be discussed here. It is, however, of interest that he considered but dismissed the possibility that carbon dioxide may promote acid synthesis (see p. 412). In the development of de Saussure's work Warburg made several positive contributions to empirical knowledge. He obtained evidence that there is a parallelism between volume decrease and dark acidification, and that in prolonged darkness, the volume begins to increase when dark de-acidification sets in. We may now attribute the decrease to the occurrence of a Wood & Werkman reaction, and the increase to the decomposition of malic acid with, so the present writer contends, the liberation of carbon dioxide.

After describing de Saussure's experiments which demonstrated that succulent plants absorb oxygen but may give off very little or even no carbon dioxide in the dark, Aubert (1892) dismissed in the following words that experiment of de Saussure which we are considering in the present section:

> *L'eminent physiologiste fait suivre l'exposé de cette série d'expériences intéressantes, des résultats, moins importants d'ailleurs, qu'il a obtenus en recherchant l'action d'un mélange d'oxygène et d'acide carbonique sur les raquettes d'*Opuntia: *il trouve que les deux gaz sont absorbés en même raison pendant la nuit, et que l'inspiration est plus prompte lorsqu'il y a de l'acide carbonique. Cette dernière observation ne nous paraît pas surprenante, étant connu le coefficient de solubilité de l'acide carbonique.*

The results of experiments which will be described in the next two papers of this series make it reasonably certain that in de Saussure's experiments, solution in the cell sap of the cactus would account for only a small fraction of the carbon dioxide absorbed in the night. In the rediscovery in 1946 at Newcastle of the effect observed by de Saussure, experiments performed by Dr Beevers showed that, while dark acidification is occurring in leaves of *Bryophyllum* placed in gas mixtures containing nitrogen, oxygen and low concentrations of carbon dioxide, carbon dioxide and oxygen were simultaneously absorbed, i.e. a negative apparent R.Q. was observed for the darkened shoots. This observation was reported at a meeting of the Society for Experimental Biology held in London in January 1947. Later, Thomas (1947, p. 269) wrote: 'There was little doubt that the CO_2 absorption related to the metabolism leading to acid production, since it had ceased when the curve for acid accumulation reached its peak. Subsequently, CO_2 was liberated by the leaves, and at a rate approximately equal to that of oxygen absorption, i.e. during this phase the measured R.Q. was unity. Since the oxygen uptake had continued at a steady rate from the beginning of the experiment, it is inferred that the respiratory oxidation of hexose was in progress throughout, but that, while acid was accumulating, the CO_2 produced was, in addition to the CO_2 absorbed from the environment, consumed in the synthesis of acid.'

One of the objectives of recent research at Newcastle has been to show that CO_2 absorption in the dark, in the first place by leaves of plants showing Crassulacean acid metabolism, only occurs when they are accumulating acid, and that the amount of absorption relates to the extent of the power the leaves possess to accumulate acid. The relation emphasized by earlier authors was that the more intense the dark acidification in air the nearer to zero is the respiratory quotient. We may now extend this generalization and assert that R.Q.'s may fall below zero when leaves possessing a high potential for acid accumulation are placed in the dark in air enriched with carbon dioxide, and that the higher the potential the greater will be the fall. The explanation offered is that, at the outset of a dark period in pure air, it is the rate of supply of respiratory carbon dioxide to the enzymic centres of acid synthesis, and not that of the supply of products of glycolysis, which limits the rate of the Wood & Werkman reaction. Since there is a CO_2 want at these centres, CO_2 feeding will inevitably result in CO_2 absorption commensurate with the maximum potential rate of acid synthesis by the leaves under the experimental conditions.

Much remains to be elucidated about gaseous exchanges and acid production. If the overall equations (*a*), (*b*) and (*c*) represent the only metabolic processes affecting gaseous exchange that occur whilst dark acidification is in progress, simple arithmetical calculation permits the evaluation from the amount of carbon dioxide fixed by leaves in pure air or in CO_2-enriched air of the amount of titratable acid (reckoned exclusively as malic acid) that should accumulate. The results obtained by Dr Beevers in 1946 (see Thomas & Beevers, 1949) and by Dr Ranson later (see Thomas & Ranson), indicate that the weight of carbon dioxide fixed is never less and sometimes decidedly greater than can be accounted for by the accumulation of titratable acid. Discussion of possible ways of accounting for the discrepancy will be deferred until the results are published. Although a final statement about overall gaseous exchanges which accompany acid production cannot yet be made, it can be confidently stated that the amount of CO_2 fixation relates to the power leaves possess to accumulate acid. The de Saussure effect, rediscovered at Newcastle, is attributable beyond reasonable doubt to reactions of the type discovered by Wood & Werkman.

III. LIGHT DEACIDIFICATION, WITH INCIDENTAL REFERENCE TO DARK DEACIDIFICATION AND LIGHT ACIDIFICATION

A. *Light deacidification as resulting from the consumption in photosynthesis of respiratory and other carbon dioxide*

If it is accepted that diurnal changes in acidity, which take place only in organs containing chloroplasts, relate predominantly to alterations in the internal concentration of carbon dioxide rather than to those of oxygen, and that acids are produced and consumed both in the light and in the dark, the occurrence of light deacidification can be satisfactorily explained on the assumption that a Wood & Werkman reaction, involving the metabolic consumption of carbon dioxide at protoplasmic centres of acid synthesis, is an essential stage in the production of malic acid. When leaves are illuminated after spending a period in the dark, such respiratory carbon dioxide as accumulated inside them in the dark or such carbon dioxide as continues to be produced will, with carbon dioxide absorbed from the atmosphere, be preferentially consumed in photosynthesis. The concentration of carbon dioxide at centres of acid synthesis will therefore fall to a very low value, and, in consequence, the rate of acid production will become very slow; indeed, it may fall to zero. The existing fragmentary experimental evidence concerning the influence of oxygen and carbon dioxide concentrations on the rate of acid consumption (see pp. 396 and 414) indicates that any accelerations resulting from the reduction of the internal carbon dioxide concentration and from the increase in the internal oxygen concentration owing to photosynthesis, will be small in comparison to the effects on acid production induced by changes in CO_2 concentration.

When the rate of consumption exceeds that of production light deacidification will set in. Acid that accumulated while the leaves were in the dark will progressively disappear from the green cells. Disappearance will be relatively fast under conditions favourable for photosynthesis because the active mass of carbon dioxide available for the Wood & Werkman reaction will then be negligibly small. The rate of light deacidification will be slower under conditions less favourable for photosynthesis. Evidence of retardations with falling light intensities is recorded in the older literature (e.g. Warburg, 1886; Astruč, 1903). Indeed, at low light intensities the supply of respiratory carbon dioxide to centres of acid synthesis may, even under natural conditions, be sufficient to raise the rate of acid production above that of consumption, i.e. to bring about light acidification. Warburg stressed the fact that acid minima, which are not maintained for many hours, are often found at midday or shortly after. This seems to imply that light acidification can occur in the afternoon and evening.

Under natural conditions, when photosynthesis is active during a bright summer morning, the supply of sugar for glycolysis at the centres of acid synthesis may never be the factor that limits the rate of the Wood & Werkman reaction. In contrast, during dark deacidification, which may be a symptom of incipient starvation, it is conceivably a fall in the rate of supply of sugar rather than a shortage of carbon dioxide which causes the rate of acid production to become slower than that of acid consumption. The charts drawn by Bennet-Clark (1933*a* and *b*) clearly show that the rate of light deacidification is normally much faster than that of dark deacidification. After briefly formulating his hypothesis Thomas (1947, p. 328) wrote: 'if relatively high concentrations of CO_2 were maintained in the interior by the unopposed respiration of green shoots in the dark, deacidification, when it sets in as a starvation phenomenon, would be a relatively slow process. Production would go on continuously, as well as consumption. It would depend upon the relative rates of these two processes whether the sap would get more acid or less acid. If, in the light, the accumulated CO_2 is quickly and completely used up by green shoots, photodeacidification would be a quick process. Acid would continue to be consumed, but it would no longer be produced.' Implicit throughout this paragraph are the assumptions that the same protoplasmic mechanisms for acid consumption and production operate in the light and in the dark, and that the occurrence of acidification or deacidification is determined not by qualitative changes in metabolism but by such quantitative changes in the continued production and consumption of acids as may result from alterations in the concentrations of reactants or of accumulating products of reactions.

B. *Values of assimilatory quotients during light deacidification in air as providing evidence that this process, like dark deacidification, is accompanied by intracellular* CO_2 *production*

Whereas during dark acidification in air R.Q.'s range from zero to unity (p. 400), during dark deacidification they are greater than unity, indeed, sometimes much greater (p. 401). Clearly in acid consumption in the dark, a greater volume of carbon dioxide is produced by the enzyme system concerned with consumption than the volume of oxygen absorbed by this system. Indeed, the present writer has suggested (p. 402) that in such acid consumption as may occur by oxidative anabolism the net absorption of oxygen may be nil, and that the consumption may tentatively be represented by the overall equation

$$2C_4H_6O_5 = C_6H_{12}O_6 + 2CO_2. \qquad (c)$$

It is admitted that acid may be simultaneously consumed in other ways, for example, by oxidative katabolism,

$$C_4H_6O_5 + 3O_2 = 4CO_2 + 3H_2O, \qquad (d)$$

or by conversion into amides, ammonium salts, etc.

The preliminary indications given by experiments done at Newcastle that the values of R.Q.'s are approximately unity when dark acidification has reached a maximum (p. 418), and the fact that the values recorded by Astruč and by Bennet-Clark (see p. 401) for dark deacidification are high, can be explained on the assumption that the normal aerobic respiration of carbohydrate is predominantly accompanied by the oxidative anabolism of acids rather than by their oxidative katabolism. Whether or not this explanation will turn out to be correct, it is clear that during dark deacidification, i.e. when acid consumption exceeds acid synthesis, there is a net CO_2 production for acid metabolism considered apart from the normal aerobic respiration of sugar.

If it is accepted that light and dark deacidification are governed by the same enzymic mechanism, it follows from (*a*) the statements made in the previous paragraph, (*b*) the hypothesis that a Wood & Werkman reaction takes part in acid production, and (*c*) long-established knowledge of gaseous exchanges in photosynthesis and respiration, that when, in illuminated leaves of plants showing Crassulacean acid metabolism, the rate of acid production is different from that of acid consumption, the assimilatory quotient (O_2/CO_2) will depart from the value of unity.

If, as usually happens in green cells illuminated in air after they have accumulated acid in the dark, acid consumption exceeds acid synthesis, there will be a net CO_2 production for acid metabolism as a whole. Unlike respiratory CO_2 production this, as has been pointed out above, may possibly be unaccompanied by a net oxygen uptake. Like respiratory carbon dioxide, however, this carbon dioxide is available for photosynthesis and may be totally consumed in this process, although, of course, some may diffuse into the outside atmosphere. In some of his experiments Aubert (1892) detected the simultaneous evolution of oxygen and carbon dioxide by leaves undergoing light deacidification. The consumption in photosynthesis of carbon dioxide produced in acid metabolism will lead to the production of an equimolecular amount of oxygen. It has long been realized that the existence of this additional internal source of carbon dioxide will result in an increase in the assimilatory quotient (A.Q.) above unity, and to the development of positive pressures when leaves undergo light deacidification in closed spaces.

It has been reported that cacti (de Saussure) and members of the Crassulaceae (Mayer, 1878) differ from most other green plants in that, when illuminated in the absence of carbon dioxide, they can give off considerable amounts of oxygen. The values of their A.Q.'s would then have to be represented by the infinity symbol. De Saussure did his work before the discovery of diurnal variations in acidity. Mayer sought experimental evidence to explain these variations. From his results he inferred the existence of a causal nexus between light deacidification and the oxygen liberated in the absence of carbon dioxide, and suggested that accumulated acids, in disappearing from leaves, yield carbon dioxide, which, by serving as a substrate of photosynthesis, spares carbon dioxide in the outside atmosphere. Mayer's conclusions will explain the results obtained by Aubert (1892), who measured the A.Q.'s of a number of illuminated green plants. Among his recorded values for succulents are 1·13 for *Pereskia*, 2·03 for *Kleinia*, 2·38–7·59 for *Opuntia tomentosa*. He stated that the range for ordinary plants is from 1·05 to 1·18, and generalized about succulent plants in these words: *Plus elles sont charnues, plus elles renferment de ces acides en réserve, plus elles dégagent d'oxygène à la lumière*. At Newcastle we are not at present concerned with the relation between the degree of succulence and the extent of diurnal variation in acidity. Aubert's virtual attribution of high A.Q.'s to relatively intense light deacidification has, however, been noted because the relation can tentatively be accounted for on the assumption that in so far as acid is consumed by oxidative anabolism, this consumption occurs with CO_2 production but without oxygen absorption.

Astruč (1903) obtained clearer evidence that a high A.Q. relates directly to a relatively high rate of acid consumption in the light. This evidence helped to establish the view that light deacidification is subjected to the influence of two general functional processes, viz. respiration and photosynthesis, and that, by the latter process, much or all of the carbon dioxide arising from the destruction of malic acid may be consumed. Astruč detached leaves of *Sedum tectorum* along a line from the centre to the periphery of a rosette, after they had accumulated acid to a maximum or near maximum value in the dark. He had previously shown that this value is generally greater and diurnal variations are wider in the older peripheral leaves than in the central ones. In each experiment he used similar leaves, and measured such CO_2 output and oxygen uptake in the dark, oxygen output in a moderately high light intensity, and dark or light deacidification, as occurred simultaneously during the experimental period. Assuming that the respiratory intensity in the light was the same as in the dark, he calculated the A.Q.'s of the illuminated leaves, by dividing the sum of the volumes of oxygen absorbed in the dark and given off in the light by the volume of respiratory carbon dioxide liberated in the dark. Both of the values recorded in Table 1 for a pair of Astruč's experiments are well above unity. With a fall in acidity of 1·1 in the central leaf the A.Q. was 1·30, which was, it is considered, significantly less than the A.Q. of 1·93 obtained for the peripheral leaf in which the acidity fell by 3·3 during the period of illumination. We note in passing that during dark deacidification in this experiment the R.Q. was greater than unity, and again recall that this indicates the liberation of carbon dioxide in the consumption of acid in molecular amounts greater than those of the oxygen absorbed by the metabolic system effecting this consumption.

Astruč's other experimental results consistently showed that the greater the fall in acidity in the light the higher was the A.Q. The existing published evidence therefore appears to be convincing that there may be a vigorous intracellular production of carbon dioxide accompanying light deacidification. This may be more vigorous than that which is attributable to dark deacidification, and not to respiration, in parallel experiments (such as those performed by Astruč), because, in the dark, continued acid production involving the fixation of carbon dioxide will reduce the net amount of carbon dioxide set free during acid metabolism. Anticipating a mode of analysis to be used by Thomas & Beevers, we may calculate acid production in the dark by the peripheral leaf in Astruč's experiment, on the assumption that no acid was produced in the light. Since acid consumption in the light was 3·3 units, the gross consumption in the dark was also 3·3 units. The net measured consumption in the dark, however, was only 0·7 unit. Hence the acid production during dark deacidification was 2·6 units.

Table 1

	Central leaf	Peripheral leaf
CO_2 output in the dark	0·30	0·32
Oxygen absorption in the dark	0·24	0·27
Oxygen output in the light	0·15	0·30
Assimilatory quotient (O_2/CO_2)	1·30	1·93
Acid number at outset	4·7	5·9
Acid number at end in the dark	4·1	5·2
Acid number at end in the light	3·8	2·6

Quantitative experiments, resembling Astruč's in some respects, have been performed at Newcastle (see Ranson) to determine whether the amount of acid consumed in light deacidification can, on the basis of the present writer's development of Bennet-Clark's hypothesis or on that of any other hypothesis, be equated to that amount of non-respiratory carbon dioxide which is simultaneously liberated by intracellular processes. For dark deacidification the comparisons to be made will be between net carbon dioxide liberation and net acid disappearance.

C. *The retardation of light deacidification and occasional induction of light acidification by* CO_2 *feeding*

If a Wood & Werkman reaction participates in acid production, it should be possible to alter the rate of production, either in the light or in the dark, by altering the amounts of carbon dioxide available at centres of acid synthesis. This argument was the starting point for some of the experiments which will be described by Thomas & Beevers. About these experiments carried out in the light, Thomas (1947, p. 328) published the following statement: 'The results of preliminary experiments performed in the summer of 1946 at Newcastle by Beevers have been reported at a meeting of the Society for Experimental Biology. He succeeded in causing acids to accumulate in *illuminated Bryophyllum* leaves, by exposing them to gas mixtures containing supernormal concentrations of CO_2, but not high enough to inhibit metabolic processes. We infer that the rate of production of acid was enhanced by raising the CO_2 concentration, and reached a value exceeding the rate of consumption. We have evidence...that CO_2 acts as a metabolite in acid production, and believe that, in the light as well as in the dark, the concentration of this gas exerts a quantitative control on acid production. There is no evidence that qualitative changes are brought about by such CO_2 concentrations as occur under natural conditions or as we have used in our experiments.'

Here, the present writer will discuss, on the basis of the statements made in the previous paragraph, some of the results obtained by certain earlier workers in their experiments on the influence of supernormal concentrations of carbon dioxide on acid metabolism in the light and in the dark. Broadly, his contention is that this influence is exerted by promoting acid production rather than, as the earlier workers thought, by retarding acid consumption.

Warburg (1886) obtained clear evidence for the leaves of several genera showing Crassulacean acid metabolism that, during the day, they lost much less acid when exposed to atmospheres containing 20–25 % carbon dioxide in some of his experiments, and 12·5 % in others, than corresponding leaves lost when exposed to pure air. In some of the experiments no light deacidification was observed in the leaves fed with carbon dioxide. For example, in one experiment *Bryophyllum* leaves were placed in ordinary air in the dark, and two comparable samples were illuminated, one in ordinary air and the other in air enriched with 25 % carbon dioxide. The values recorded in Table 2 indicate that light deacidification occurred in air, but not in the enriched air. No exception can be taken to Warburg's conclusion that carbon dioxide retarded light deacidification. The present writer maintains that it did this, in the main, by promoting the Wood & Werkman reaction under conditions of an abundant supply of products of glycolysis, and, consequently, by accelerating the rate of acid synthesis.

Table 2

	Acid values of *Bryophyllum* leaves
After a period in the dark in air	91
After the same period in the light in air	21
After the same period in CO_2-enriched air	90

Table 3

	Acid values of *Bryophyllum* leaves
After a period in the dark in air	43·7
After the same period in the light in air	17·0
After the same period in the light in (air + 25 % CO_2)	62·2*

* Average of three values: 66·7, 58·7 and 61·2.

From the results of other experiments Warburg arrived at the highly important conclusion that *Öfters vermehrt sich der Säuregehalt etwas am Tage in kohlensäurenreicher Luft*. The results of one of these experiments are recorded in Table 3. Since Warburg measured the acid values of each of the three leaves fed with carbon dioxide, we have some idea of the sampling errors in his experiments. If, but only if, no dark deacidification occurred in the leaf which was kept in the dark in air, Warburg's conclusion was justifiable, and his experiments would provide evidence of light acidification as a result of CO_2 feeding. But it is possible that the leaf used in the experiment in air in the dark had, at the outset, an acid value even greater than 62·2. However, the experimental results which we have obtained at Newcastle during the period 1946–48 support his conclusion.

Warburg stated the possibility that the effects of retarded light deacidification and, sometimes, of acidification in the light, in atmospheres enriched in carbon dioxide, might conceivably be explained by attributing to this gas a power to stimulate acid building and so to cause acid production to compensate for acid consumption. But for reasons which must have appeared cogent to his contemporaries, he rejected this explanation. Amongst other matters, he considered critically de Saussure's contention that, in the dark, *Opuntia* retains carbon dioxide produced in respiration. He admitted the validity of de Saussure's facts, but, because he did not develop de Saussure's conclusions to explain his own observation that carbon dioxide retards light deacidification, missed the opportunity of anticipating by 60 years the discovery of CO_2 fixation during the metabolic production of acids. From his own experimental results he concluded that CO_2 feeding only influences acid metabolism in the light and in cells containing chloroplasts. From what was stated on p. 406 it is clear that at least the first of these conclusions is not now acceptable. Having arrived at them, however, Warburg found himself in agreement with de Vries who had earlier stated that light accelerates a process of deacidification which is itself unconnected with light. This statement is still acceptable, for we may regard the production and consumption of acids as going on independently of light, and the acceleration of deacidification by light as occasioned by the retardation of production through the removal by illuminated chloroplasts of carbon dioxide, one of the substrates which is essential for acid synthesis.

Astruč (1903) made a number of experiments with leaves of *Sedum tectorum*, and compared light deacidification in atmospheres containing carbon dioxide with that occurring in air. The results of his preliminary experiment are recorded in Table 4. He concluded that *Ces premières données montrent déjà que l'anhydride carbonique, même à faible dose, entrave fortement la destruction de l'acide malique, chez les Crassulacées.* The two points to notice are the retarding effect of even 5% CO_2 on light deacidification, i.e. a much lower concentration than Warburg had used in his experiments, and the unacceptable conclusion that carbon dioxide necessarily acts by strongly retarding acid consumption. Elsewhere in his paper in discussing other matters, Astruč had written: *Mais l'acidification et la désacidification sont, en somme, deux phénomènes coexistants, se produisant constamment, et le terme final observé n'est que la resultante des deux actions contraires.* Astruč clearly meant that acid production and consumption could occur continuously under all conditions, in the light and the dark. Accordingly, an alternative conclusion he might have drawn from the experimental results recorded above was that carbon dioxide, even in concentrations as low as 5%, can promote acid production. From other experimental results he came, however, to the opposite conclusion that carbon dioxide can retard acid production. He compared the amounts of acids accumulating overnight in leaves placed in a series of gas mixtures containing different percentages of carbon dioxide. His results indicated retardations when the CO_2 concentrations exceeded 10%: but, as Thomas & Ranson will report, the effects induced by relatively high CO_2 concentrations, such as were used in much of the earlier work, may differ in some respects from those observed in concentrations sufficiently low not to retard the Wood & Werkman reaction.

Table 4

	Relative weights of malic acid
At the outset in the morning	6·0
After illumination until the evening:	
In air	4·4
In 5% CO_2	5·8
In 20% CO_2	5·8
In 50% CO_2	5·8
In 100% CO_2	6·0

In some of his experiments on light acidification Wolf (1931) used CO_2 concentrations of 30 and 50%. It may well be that, exposed for many hours to such high concentrations of this essential substrate for acid synthesis, enzyme mechanisms concerned with both the production and the consumption of acids may be partially inhibited. The possible multiple effects of carbon dioxide on acid metabolism may profitably be compared with those exerted on photosynthesis and respiration by high concentrations of this gas.

Of much interest are the comparisons Wolf made between the amounts of light deacidification over a fixed period in leaves kept in normal air and in leaves kept in CO_2-free air. For leaves of *Sempervivum glaucum* and of *Bryophyllum calycinum* his results indicated that light deacidification was unmistakably faster in CO_2-free air. Such results would clearly suggest the possibility that, even when photosynthesis is active under natural conditions, chloroplasts do not consume all the carbon dioxide absorbed from the outside atmosphere. Some may find its way to centres of acid synthesis and be consumed in the Wood & Werkman reaction.

D. *The influence of* CO_2 *feeding on acid consumption in the dark and therefore on acid consumption in the light*

Warburg (1886) carried out experiments to determine whether carbon dioxide hindered the dark deacidification of *Bryophyllum* leaves. He used leaves which had accumulated acid overnight and placed them for a further number of hours in the dark, some in pure air and some in air enriched with carbon dioxide. Virtually he prolonged the dark period that in nature night imposes. Because de Vries had already shown that deacidification is favoured by raising the temperature, Warburg did some of his experiments at 35° C. The results of one of these experiments is recorded in Table 5. In none of his experiments, excepting when the leaves suffered injury, was dark deacidification affected by carbon dioxide concentrations of 12 or of 25%. It remains to be determined whether acid production in leaves fed with carbon dioxide under these conditions is negligibly small. If it is, the conclusion to be drawn from Warburg's experiment is that carbon dioxide in concentrations of 25% or less does not, over a period reckoned in hours, retard the rate of acid consumption.

Table 5

	Acid value of leaves
At the outset in the morning	48·1
After 24 hr. in the dark in air	19·0
After 24 hr. in the dark in 25% CO_2	19·4

Much more recently Wolf (1931) obtained some evidence, however, that carbon dioxide in concentrations of 20% or more may retard dark deacidification. He floated, on water, three half-portions of *Bryophyllum* leaves which had previously reached their acid maximum during a preliminary dark period of 17 hr., and kept each portion in a different gas mixture in the dark for a further period of 20 hr. To promote dark deacidification he carried out his experiment at 37° C. As a percentage of the initial acid value deacidification in (air + 30% CO_2) was 12, in (air + 20% CO_2 + 10% nitrogen) it was 14, and in (air + 30% nitrogen) it was 23·3. As during this experiment it may well be that the rate of glycolysis rather than the rate of CO_2 supply limited the rate of acid production, Wolf's conclusion that carbon dioxide, in the high concentration which he used, retarded acid consumption may be valid, but it is also possible that CO_2 feeding resulted in slower deacidification because it promoted the Wood & Werkman reaction and, in consequence, acid synthesis. Wolf attributed the retarding effect to the partial inhibition by carbon dioxide of oxidation enzymes (e.g. malic dehydrogenase) concerned with the consumption of acids. Wolf's explanation is a reasonable one, and to support it one might cite physiological evidence of retarded respiration under the influence of lower CO_2 concentrations and of disordered respiration, leading to CO_2 zymasis (see Thomas, 1925), induced by higher concentrations. But, as Beevers (1947) has found, it is difficult to prove by experiments *in vitro* that carbon dioxide, in concentrations even higher than 80%, impairs the activity of oxidation enzymes, excepting in so far as the pH of the medium is reduced when the carbon dioxide dissolves in it.

We note that Wolf's conclusions differed from Warburg's. The question is still unanswered whether or not rising carbon dioxide concentrations progressively retard acid consumption in dark deacidification, and, therefore, as Wolf argued, acid consumption in light deacidification also. This question must be answered before an explanation of the causes of diurnal variation in acidity can approach completion. The CO_2 concentrations used in experiments at Newcastle have, as a rule, been less than 10%. In the next two papers of this series it will be provisionally assumed that in these concentrations any retarding effect of carbon dioxide on acid consumption, due to enzyme inhibition or mass action, may be neglected.

E. *Volume changes in enclosed spaces and assimilatory quotients when leaves showing Crassulacean acid metabolism are illuminated in CO_2-enriched air*

In his extension of de Saussure's experimental investigations (see p. 410) Warburg enclosed leaves of *Bryophyllum* and other genera in vessels containing either ordinary air or atmospheres enriched with 25% carbon dioxide. The vessels were fitted with manometer side-arms. He observed a parallelism between volume increase and acid decrease during the day. From his results he concluded that both of these magnitudes were greater when the leaves were illuminated in air. From the explanation which has already been given on p. 410 of volume increase when leaves are undergoing light deacidification in air in closed spaces, it follows that if CO_2 feeding retards light deacidification by promoting acid production, this increase would be less in leaves fed with carbon dioxide. If acid is produced by the Wood & Werkman reaction, and consumed by oxidative anabolism in the way tentatively suggested by the present writer, the net turn-over of carbon dioxide would be nil, and an enclosed volume would remain unchanged, during a period of illumination in which the original acidity of leaves was maintained. Clearly, if these arguments are well founded, the enclosed volume should decrease if CO_2 feeding leads to light acidification.

The statements made above may be put in another way. If, in spite of CO_2 feeding, light deacidification occurs the A.Q. will be greater than unity, but will progressively approach unity as deacidification progressively diminishes. If CO_2 feeding leads to light acidification, the A.Q. will be less than unity. The more acid production, involving CO_2 fixation, exceeds acid consumption, possibly by oxidative anabolism and with the liberation of carbon dioxide, the lower will be the A.Q. Evidence has been obtained which supports these statements (see Ranson).

GENERAL CONCLUSIONS

A. *Summarized conclusions about Crassulacean acid metabolism*

In most, if not all, living cells of plants, there may possibly be present enzymic systems which are concerned with the production and consumption of acids. If this assumption is conceded, it can be argued that in plants showing easily detectable Crassulacean acid metabolism both systems have a relatively high activity, and, moreover, that the system which governs production is *potentially* the more active of the two. With the necessary substrates all present in ample supply the rate of acid production would then exceed that of its consumption, and acids would accumulate. If one of the essential substrates is missing or in very deficient supply, the accumulated acids already present will tend to disappear. Diurnal fluctuations in acidity would therefore tend to occur if the available concentration of an essential substrate varied during a 24 hr. period under natural conditions.

Wood & Werkman's discovery in their work on bacteria, and the results of further work on mould fungi and animal cells, suggested the possibility that carbon dioxide is one of the substrates essential for the synthesis of acids by green plants, and that it combines with pyruvic acid (or a phospho-derivative thereof), a product of glycolysis, to form dicarboxylic acids containing four carbon atoms. In the formation of tricarboxylic acids there may be further fixations of carbon dioxide.

Now in the green parts of plants, but only in these (and it is only in these that Crassulacean metabolism is observed), the internal concentration of carbon dioxide is a periodic variable. It tends to decrease in the light when photosynthesis is active and to increase when photosynthesis has stopped in the dark, or, in failing light, is not sufficiently active to mask normal aerobic respiration completely.

On a bright summer's day, therefore, the rate of acid production will fall to a low value. If the potential powers of enzymes concerned with the consumption of acid remain unchanged, accumulated acid will disappear from the green cells, i.e. light deacidification will occur.

After dusk, in the green cells well stocked with carbohydrate after a day of photosynthetic activity a good supply of carbon dioxide, coming from the normal aerobic respiration of sugar, will eventually become available at centres of acid synthesis. These centres will also be well supplied with products of glycolysis. Accordingly, the rate of acid production will rise. When it exceeds that of consumption, acid will accumulate in the cells, i.e. dark acidification will take place.

Bennet-Clark (1933*a*), referring to plants that show Crassulacean acid metabolism, states: 'This group probably includes all succulent plants with the exception of certain Centrospermae. Many non-succulent plants are of this type, the most noteworthy being the Orchidaceae and Bromeliaceae. Many plants containing malic acid in which diurnal periodicity has not been observed may of course have a similar type of metabolism with a fluctuation so small that it does not exceed the sampling errors.' Among the possible explanations of such small fluctuations, if they occur, is that the enzymic systems concerned with acid metabolism have only feeble powers of bringing about the Wood & Werkman and allied reactions. Although in some plants succulence, by favouring the retention of respiratory carbon dioxide, may promote the Wood & Werkman reaction, it is clearly not a necessary factor. It is possible that in the Orchidaceae and Bromeliaceae the enzyme system concerned with CO_2 fixation is much more active than the corresponding system in succulent plants of 'certain Centrospermae'.

B. *Summarized statement of evidence cited in support of some of the above conclusions*

(1) That recorded values of the respiratory quotients during dark acidification in plants showing Crassulacean acid metabolism in ordinary air have been increasingly below unity when the intensity of acidification has been increasingly high is attributed to the more vigorous fixation of respiratory CO_2, which occasions the more vigorous acid production. It is at present assumed that oxygen uptake is not directly affected by acid metabolism.

(2) The near coincidence of the troughs of CO_2 output curves with the peaks of progress curves for changes in the acid content of leaves that are respiring in the dark, is attributed to the vigorous occurrence of the Wood & Werkman reaction when the rate of acid production exceeds that of acid consumption, i.e. when the net effect is dark acidification.

(3) De Saussure reported that, of the dark, *Opuntia* may simultaneously absorb carbon dioxide and oxygen from a gas mixture containing both of these gases. New interest attaches to the de Saussure effect since it was rediscovered in Newcastle in 1946. Convincing evidence will be published in the next two papers of this series that in the dark such simultaneous absorption occurs during dark acidification, and only during dark acidification. The R.Q.'s are then negative. This is the best evidence so far obtained at Newcastle that the Wood & Werkman reaction takes place in green plants. In plants showing Crassulacean acid metabolism such evidence is obtainable without using carbon dioxide containing tracer carbon, which has not yet been available in Newcastle. When it is available we propose to use it for determining whether carbon dioxide can be fixed by leaves or fruits which accumulate malic acid but do not clearly show the Crassulacean type of metabolism, and by genera of the Crassulaceae and Cactaceae during those periods of the year in which diurnal variations in acidity are no longer detectable. Clearly this may be because both production and consumption have stopped, or because their rates are equal. In the latter event tracer carbon should be found in carboxylic groups when leaves are fed in the dark with carbon dioxide containing heavy carbon.

(4) In 1945 the present writer concluded that, if light deacidification results from the retardation of acid production owing to a deficiency in carbon dioxide, it should be possible to slow it down by promoting acid production through CO_2 feeding. Convincing evidence that this happens was obtained by work performed during the period 1946–8, after an unsuccessful beginning in September 1945. The evidence will be presented in the next two papers of this series. Indeed, production has been sufficiently promoted to exceed consumption, i.e. to induce light acidification. The earlier literature has provided much supporting evidence, which is reported in the present paper. Prior to the work at Newcastle the results of such experiments have always been interpreted by attributing to carbon dioxide the power to retard acid consumption. Clearly the explanation has always been inadequate for those experiments in which light acidification was observed.

C. *Summarized conclusions about gaseous exchanges during dark acidification and light and dark deacidification*

(1) In the dark, the net gaseous exchange observed will be determined by the relative rates of the normal aerobic respiration of carbohydrate, of acid production and of acid consumption. For aerobic respiration, considered in isolation, the value of the quotient

volume of CO_2 *produced/volume of oxygen absorbed* is unity. If the overall equation (*b*) written on p. 398 is correct, this quotient for acid production, considered in isolation, is indeterminable, being a negative value divided by zero. If the overall equation (*c*) written on p. 409 is correct, this quotient for acid consumption, considered in isolation, must be represented by the infinity sign. This analysis makes it easy to understand why the value of the quotient *volume of* CO_2 *escaping from a leaf/volume of oxygen absorbed by the leaf*, i.e. the measured respiratory quotient, is less than unity, and may be zero, when, during dark acidification, acid production exceeds acid consumption; and why it is greater than unity, sometimes reaching values more than 2, when, during dark deacidification, acid production falls below acid consumption possibly because of a reduced rate of glycolysis resulting from prolonged starvation conditions.

(2) If experiments now in progress prove conclusively what appears to be probable from results so far obtained at Newcastle, that the measured R.Q. is unity when acid accumulation in the dark is temporarily maintained at a maximum value, i.e. when the rates of production and consumption of acid are equal, strong evidence will have been procured that equimolecular amounts of carbon dioxide are fixed in the production and liberated in the consumption of a given amount of acid. The pair of equations (*b*) on p. 398 and (*c*) on p. 409 would clearly satisfy such a relation, and it is contended fit the known facts better than any other pair that might be suggested at present.

(3) Equation (*c*) summarizes a modified form of Bennet-Clark's hypothesis that malic acid is consumed by an anabolic rather than by a katabolic process. Since the subject of the acid metabolism of plants was brought in the orbit and under the influence of the idea of CO_2 fixation in acid production, the logical development of arguments has led to the conclusion, differing from Bennet-Clark's, that carbon dioxide is liberated during this anabolic consumption. We have seen that the high R.Q.'s found during dark deacidification can be explained, if there is a net CO_2 output for total acid metabolism superimposed upon the gaseous exchange of normal aerobic sugar respiration. *A priori* biochemical arguments also incline one to the view that in the consumption of carboxylic groups, decarboxylation will play an essential part. The results of studies on the decarboxylases of higher plants will require much consideration before detailed analysis of the partial reactions in acid consumption can be attempted. It is not inconceivable that the production and consumption of acids are truly reversible processes. There is also the clear possibility, summarized in the scheme on p. 402, that the final stage in oxidative anabolism is glycolysis in reverse.

(4) It has long been accepted that acids are always consumed, in the light and the dark, by the same enzymic system. If acid production virtually ceases in illuminated green cells of plants showing Crassulacean acid metabolism, the net gaseous exchange in the light will be determined by the relative rates of photosynthesis, of normal respiration and of acid consumption. Clearly, analysis along the lines of para. (1) above will lead to the conclusion that, if acid consumption is accompanied by CO_2 production, the value of the quotient *volume of oxygen evolved/volume of* CO_2 *absorbed* by an illuminated leaf undergoing light deacidification in ordinary air will be greater than unity. The older literature provides evidence supporting this conclusion. Additional evidence has been obtained at Newcastle from measurements of the gaseous exchange of leaves illuminated in air enriched with carbon dioxide (see Ranson). So far every prediction has been fulfilled. With light deacidification progressively retarded by CO_2 feeding the measured A.Q. falls in the

direction of unity; with light acidification induced by CO_2 feeding, the measured A.Q. becomes less than unity. If it can be proved that with certain conjunctions of CO_2 concentration, light intensity, and temperature, the A.Q. is maintained at unity when the amount of accumulated acid remains unchanged (i.e. when the rate of production equals the rate of consumption) further evidence (see para. (2) above) will be forthcoming that the pair of overall equations

$$C_6H_{12}O_6 + 2CO_2 = 2C_4H_6O_5, \quad 2C_4H_6O_5 = C_6H_{12}O_6 + 2CO_2,$$

provide a satisfactory tentative summary of the views put forward in the present paper for the production and consumption of acids. These equations will have to be modified or supplemented if significant differences are found between measured values and the values calculated from the above equations (i) for net CO_2 absorption per unit amount of acid accumulating during dark or light acidification, and (ii) for net CO_2 liberated per unit amount of accumulated acid which disappears during light or dark deacidification. Since the measured and calculated values to be recorded in the next two papers of this series are sometimes widely different, no confidence is yet felt that a statement approaching finality can yet be made about the overall gaseous exchanges accompanying acid metabolism in green plants. But it is confidently asserted that the experimental results obtained by Dr Beevers and Dr Ranson at Newcastle, coupled with those obtained by earlier workers, prove that carbon dioxide is metabolically fixed in acid production and metabolically liberated in acid consumption. The earlier work has been extensively considered in this introductory paper, which, it is hoped, will provide an adequate background for the discussion of the results of experiments already performed and of those still in progress at Newcastle to fill in details of the picture.

REFERENCES

Astruč, A. (1903). Recherches sur l'acidité végétale. *Ann. Sci. nat.* (*Bot.*), Ser. 8, **17**, 1.

Aubert, M. E. (1892). Recherches sur la respiration et l'assimilation des plantes grasses. *Rev. gén. Bot.* **4**, 203.

Barron, E. S. Guzman (1943). Mechanism of carbohydrate metabolism. An essay on comparative biochemistry. *Advances in Enzymology*, **3**, 149.

Beevers, H. (1947). Some experiments on the effect of carbon dioxide on the acid metabolism of *Bryophyllum*. Ph.D. Thesis, Part II. Durham University.

Bennet-Clark, T. A. (1930). The metabolism of succulent plants. *Rep. Proc. Vth Int. Bot. Congr.* p. 425.

Bennet-Clark, T. A. (1932). The respiratory quotients of succulent plants. *Sci. Proc. R. Dubl. Soc.* **20**, 293.

Bennet-Clark, T. A. (1933*a*). The role of the organic acids in plant metabolism. Part I. *New Phytol.* **32**, 37.

Bennet-Clark, T. A. (1933*b*). The role of organic acids in plant metabolism. Part II. *New Phytol.* **32**, 128.

Bennet-Clark, T. A. (1933*c*). The role of the organic acids in plant metabolism. Part II. *New Phytol.* **32**, 197.

De Saussure, T. (1804). *Recherches chimiques sur la Végétation.* Paris.

De Vries, H. (1884). Über Säurebildung der Fettpflanzen. *Bot. Ztg*, **42**, 337.

Gollub, M. & Vennesland, B. (1947). Fixation of carbon dioxide by a plant oxalacetate carboxylase. *J. Biol. Chem.* **169**, 233.

Gustafson, F. G. (1925). Diurnal changes in the acidity of *Bryophyllum calycinum*. *J. Gen. Physiol.* **7**, 719.

Krampitz, L. O. & Werkman, C. H. (1941). The enzymic decarboxylation of oxaloacetate. *Biochem. J.* **35**, 595.

Kraus, G. (1883). Über Stoffwechsel bei den Crassulaceen. *Abh. naturf. Ges. Halle*, **16**, 393.

Krebs, H. A. (1943*a*). Carbon dioxide assimilation in heterotrophic organisms. *Ann. Rev. Biochem.* **12**, 529.

KREBS, H. A. (1943*b*). The intermediary stages in the biological oxidation of carbohydrate. *Advances in Enzymology*, **3**, 191.

MAYER, A. (1878). Über die Saurestoffausscheidung einiger Crassulaceen. *Landw. Versuchsst.* **21**, 277.

OCHOA, S. (1945). Isocitric dehydrogenase and carbon dioxide fixation. *J. Biol. Chem.* **159**, 243.

PUCHER, G. W., LEAVENWORTH, C. S., GINTER, W. D. & VICKERY, H. B. (1947*a*). Studies in the metabolism of Crassulacean plants: changes in the composition of *Bryophyllum calycinum* during growth. *Plant Physiol.* **22**, 1.

PUCHER, G. W., LEAVENWORTH, C. S., GINTER, W. D. & VICKERY, H. B. (1947*b*). Studies in the metabolism of Crassulacean plants: the effect upon the composition of *Bryophyllum calycinum* of the form in which nitrogen is supplied. *Plant Physiol.* **22**, 205.

PUCHER, G. W., LEAVENWORTH, C. S., GINTER, W. D. & VICKERY, H. B. (1947*c*). Studies in the metabolism of Crassulacean plants: the diurnal variation in organic acid and starch content of *Bryophyllum calycinum*. *Plant Physiol.* **22**, 360.

RABINOWITCH, E. I. (1945). *Photosynthesis*. New York.

RANSON, S. L. Further experiments on light deacidification and light acidification in *Bryophyllum*. (In preparation.)

RICHARDS, H. M. (1915). Acidity and gas interchange in Cacti. *Publ. Carneg. Instn*, no. 209.

RUHLAND, W. & WETZEL, K. (1931). Zur Frage der Aepfelsäurebildung in Crassulaceen. *Planta*, **15**, 567.

THODAY, D. & RICHARDS, K. M. (1944). Acid metabolism and respiration in succulent Compositae. II. Respiration during starvation in *Kleinia radicans*. *Ann. Bot., Lond.*, N.S., **8**, 189.

THOMAS, M. (1925). The controlling influence of carbon dioxide. V. *Biochem. J.* **19**, 927.

THOMAS, M. (1947). *Plant Physiology*, Chapter XV. London.

THOMAS, M. & BEEVERS, H. (1949). Evidence of CO_2 fixation in *Bryophyllum* and the study of diurnal variation of acidity in this genus. *New Phytol.* **48**, 421.

THOMAS, M. & RANSON, S. L. Further evidence of CO_2 fixation and new evidence of CO_2 liberation in plants showing Crassulacean acid metabolism. (In preparation.)

WARBURG, O. (1886). Über die Bedeutung der organischen Säuren für Lebensprozess der Pflanzen. *Unters. bot. Inst. Tübingen*, **2**, 53.

WERKMAN, C. H. & WOOD, H. G. (1942). Heterotrophic assimilation of carbon dioxide. *Advances in Enzymology*, **2**, 135.

WETZEL, K. (1931). Beiträge zur Kinetik der Carboxylasewirkung, u.s.w. *Planta*, **15**, 697.

WOLF, J. (1931). Beitrag zur Kenntnis des Säurestoffwechsels succulenter Crassulaceen. *Planta*, **15**, 572.

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PHYSIOLOGICAL STUDIES ON ACID METABOLISM IN GREEN PLANTS

II. EVIDENCE OF CO_2 FIXATION IN *BRYOPHYLLUM* AND THE STUDY OF DIURNAL VARIATION OF ACIDITY IN THIS GENUS

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(With 2 Figures in the Text)

The experiments to be described in the present paper were designed to determine whether or not light deacidification and dark acidification in green leaves showing Crassulacean acid metabolism could be influenced by feeding the leaves with carbon dioxide. It was argued (see Thomas, 1947, 1949) that, if the Wood & Werkman reaction participates in the production of titratable acid, CO_2 feeding in the light should retard light deacidification and, indeed, might occasion light acidification. The experiments performed to test this argument are described in § III. Owing to the fact that carbon dioxide is consumed in photosynthesis, it appeared to be unlikely that direct evidence of the utilization of this gas in the production of acid would be easily obtained from experiments carried out in the light. Accordingly, we sought direct evidence for such utilization by feeding leaves with carbon dioxide in the dark. The evidence obtained is presented and discussed in § IV.

To prepare the way for future work, some preliminary attention has been given to certain other matters relating to Crassulacean acid metabolism. For example, in § II, making some provisional assumptions, we have approximately evaluated the total production or consumption of titratable acid over stated periods in the light and in the dark; and in § III the values of respiratory quotients during dark acidification and dark deacidification are considered.

Our first requirement was that we should have a sufficient supply of leaves showing wide fluctuations of acidity under alternating conditions of light and darkness. Of plants known to have well-marked Crassulacean acid metabolism, the only species in good supply at Newcastle in the summer of 1945 was *Geranium pratense*. In the list given by Bennet-Clark (1933*a*) it is stated that Kraus had found increases of 700% overnight in the titratable acidity of the leaf sap of this plant. Preliminary experiments showed that the leaves of the plants at our disposal were acid, but we could detect no fluctuation in acidity in late August 1945. It was clear, therefore, that we should not be able to begin our experiments until the spring of 1946. A few plants of *Bryophyllum calycinum*, classical material for the investigation of Crassulacean acid metabolism, had survived the war in the heated greenhouse at this Department's garden. From these plants the departmental gardener (Lieut. C. W. W. Henderson), after he returned to Newcastle from abroad, raised a crop which had attained moderate dimensions by May 1946, and, managing to overcome certain difficulties, supplied our minimum needs during the summer of that year. We express our thanks to him for his assistance. The work continued from May until early September, when one of us (H. B.) left Newcastle to take up an appointment elsewhere.

The principal results of our investigation were reported verbally at a meeting of the Society for Experimental Biology held in London in January 1947 and in writing later in the year (Thomas, 1947). Extracts from this preliminary written report have been given in the first paper of this series (Thomas, 1949).

I. EXPERIMENTAL METHODS AND EXPRESSION OF RESULTS

A. *Plant material*

In the crop raised by the spring of 1946 there were two distinct varieties of *Bryophyllum calycinum*. In most of our experiments we used leaves from only one variety, but in others, owing to shortage of material, samples containing both varieties, making the samples as nearly alike as possible. Leaves were never in sufficient supply at any one time for carrying out a large-scale experiment from the results of which differences having clear statistical significance might have been obtained. We shall, however, attach weight to the fact that similar results were obtained in a number of experiments performed on small samples of leaves at different times in the season.

The results of our preliminary experiments confirmed those of earlier workers (see Bennet-Clark, 1933*a*) that the widest fluctuations in acidity are shown, not by the young leaves at the top of the shoot nor by the small old fleshy ones at the base, but by the larger fully developed leaves in between. Accordingly, for most of our experiments leaves below the second pair on the stem were selected. Opposite leaves were removed from a number of plants and taken to the laboratory where their petioles were freshly cut before placing them in water. Before the sample was used for an experiment a test was made on one or two of the leaves to determine the approximate extent to which acidity would fluctuate with alternating dark and light periods. Like earlier workers we found that the amount of dark acidification usually related to the average illumination of the leaves before putting them in the dark. High illumination has a dual effect: it augments light deacidification and also, by promoting photosynthesis, the accumulation of carbohydrate in relatively higher concentrations. Such leaves, when placed in the dark, will therefore have a relatively low acid content and a higher capacity for accumulating acids, since these are derived from carbohydrate. Before beginning some of our experiments, we strongly illuminated the detached leaves brought into the laboratory and thus increased their capacity for dark acidification.

B. *Apparatus and experimental conditions*

In our earlier experiments the leaves, with their petioles dipping into water, were placed in desiccator vessels filled with inlet and outlet tubes for the passage of air or of a gas mixture.

Later, a method providing superior illumination for the leaves was used (Beevers, 1947). Their petioles dipped into water contained in small tubes which were fixed to a glass rod bent to one side of a hollow glass cylinder, 3 ft. long and 4 in. in diameter. The base of each cylinder narrowed to a gas intake, and a rubber stopper with a gas outlet closed the top. The bent glass rod fitted into the stopper; when this was taken out the leaves could be removed for acid estimation. In a single experiment several such cylinders were assembled around a source of light so that the leaves, supported in natural positions, and with their upper surfaces nearest this source, were equally illuminated. During the dark periods the cylinders were covered with black paper.

Some of the gas mixtures were made up in a large metal gasometer from air and cylinder CO_2, or from cylinder oxygen and CO_2, and driven through the containers at slight pressure. In the other experiments we used gas mixtures of oxygen and carbon dioxide supplied in cylinders by the British Oxygen Company.

Until the end of June 1946 the experiments were performed at a temperature of 20° C.; for the rest of that summer at fluctuating laboratory temperature. Illumination was provided by high-wattage electric bulbs with reflectors.

C. *The determination and expression of acidity*

In order to facilitate the liberation of acid from the tissues the weighed leaf or leaves were sliced before plunging them into 100 c.c. of boiling water. Carbon dioxide was expelled from the extract by continuing the boiling for 15 min. This period was shown to be long enough for this purpose, even after experiments in which leaves had been exposed to relatively high concentrations of carbon dioxide. The CO_2-free extract was then titrated with decinormal sodium hydroxide, with a few drops of phenolphthalein as indicator. The end-point was fairly distinct.

Most of the arguments in the present paper are based on relative and not absolute changes in acidity. Accordingly the results of analysis have been expressed as titratable-acid numbers.

The titratable-acid number, which we shall represent by the symbol T.A.N., is the number of c.c. of decinormal sodium hydroxide required to neutralize the acid contained in the boiled residue and liquid originating from 100 mg. of leaf tissue. When required, the absolute acid content (reckoned as malic acid) in a known weight of leaves can, of course, be calculated.

It is realized that citric, isocitric and other acids (see Pucher, Leavenworth, Ginter & Vickery, 1947*b*) are always present with malic acid in *Bryophyllum*, but in much smaller amounts. Fluctuations in titratable acidity are, therefore, mainly owing to the production and consumption of malic acid. For the present, in order to simplify the analysis of results, we shall assume that malic acid metabolism is the sole cause of such fluctuations. In the present paper the possible occurrence of non-consumable acid (e.g. oxalic acid) will not be considered.

D. *Gas analyses and the expression of results*

All gas analyses were performed with the Ambler apparatus. The primary data give the percentage carbon dioxide, oxygen and nitrogen present at the time of analysis. This is all that is required for stating the gas conditions in describing the experiments in § II of the present paper.

The experiments on gaseous exchanges, described in § III, were all done in systems closed to the outside atmosphere. The volume of the gas mixture around the leaves was constant, but the pressure must have varied considerably. The data recorded in Tables 7, 8 and 9 indicate that at first the pressure decreased sharply because carbon dioxide and oxygen were simultaneously absorbed. Later when carbon dioxide was evolved, but at a slower rate than that at which oxygen was absorbed, the pressure still decreased but less sharply. Towards the end of the experiments, after a short period of equal rates, carbon dioxide was given off at a faster rate than that at which oxygen was absorbed. Accordingly, the pressure then increased. Pressure changes during dark acidification and dark deacidification were recorded by earlier workers (see Thomas, 1949, p. 446).

For the evaluation of real respiratory quotients between any two times t_0 and t_1 (see Tables 7, 8 and 9) and of the absolute amounts of carbon dioxide output or uptake, or of oxygen uptake (see Figs. 1 and 2 and Table 11) we have used Dr S. L. Ranson's methods of computation. These will be explained by Thomas & Ranson in the next paper of this series.

It is sufficient to state here (*a*) that these computations are based on the primary fact that the amount of nitrogen present in the closed systems remains constant over the period t_0 to t_1 irrespective of any pressure changes, and (*b*) that in the calculation of absolute amounts of absorption or output over a whole period during which gas has been removed for analysis at successive intervals, allowance must be made for each volume of gas removed.

II. DARK ACIDIFICATION AND LIGHT DEACIDIFICATION IN DETACHED *BRYOPHYLLUM* LEAVES PLACED IN ORDINARY AIR

A. *Experimental*

In the middle of May 1946 considerable diurnal fluctuations were occurring in the values of T.A.N. for leaves attached to the stems of whole *Bryophyllum* plants growing in the greenhouse. For example, in Exp. I (11 May), after the plants had been in the dark for 12 hr., such leaves had an average T.A.N. of 90. This value fell to 12 after 12 hr. of illumination. In T.A.N. units light deacidification was 78.

Fluctuations of a similar order occurred in detached leaves under controlled laboratory conditions. In Exp. II (15 May) plants were kept in the dark in the greenhouse for 12 hr. The average value of the T.A.N. for the leaves reached 88. Leaves were then cut off and brought to the laboratory. They were all illuminated strongly for 10 hr. Analysis of one sample showed that the average T.A.N. had fallen to 16. This considerable fall (62 T.A.N. units) suggested that, during the summer, we should find no difficulty in inducing light deacidification in detached leaves exposed to artificial illumination. The remaining leaves were all placed in the dark for 12 hr. Analysis of a sample showed that during this period the average T.A.N. rose from 16 to 56. This marked rise (40 T.A.N. units) indicated that, for the remainder of the season, we should be able to induce dark acidification in detached leaves under laboratory conditions. The remaining sample was then illuminated for 10 hr. The average T.A.N. fell from 56 to 9. A second light deacidification of 47 T.A.N. units had been induced in leaves during a 10 hr. period beginning 22 hr. after they had been detached from the plants growing in the greenhouse. The leaves seemed to be quite healthy at the end of the experiment. We therefore felt confident that similar experiments of even longer duration might be planned for detached leaves, and that the fluctuations in the values of T.A.N. would not depart greatly from those that would be shown in leaves attached to whole plants kept under comparable conditions in the greenhouse.

B. *Theoretical*

Observations such as we have described in § II A have often been made before, but they can now be interpreted in a new way (cf. Thomas, 1947, 1949).

The hypothesis is that in detached as well as in attached leaves, such respiratory carbon dioxide as is produced in the dark by the normal aerobic oxidation of carbohydrates has at least two fates: (*a*) escape and (*b*) metabolic consumption in the synthesis of di- and tricarboxylic acids. The experimental results reported in § II A suggest that, in mid-May 1946, (*b*) was considerable. We did not measure CO_2 output in these experiments. That it may have been low, or even zero, whilst acidification was most active is clear from the results of many earlier researches on the respiration rates and respiratory quotients of succulents, and from our later experiments (see § IV).

In detached as well as in attached illuminated leaves respiratory carbon dioxide has at least three fates: (*a*) and (*b*) above, and (*c*) consumption in photosynthesis. It is well established that in highly illuminated green leaves of all kinds of plants the rate of (*c*) may be so high that respiratory carbon dioxide escapes only at a low rate or not at all, and that considerable additional quantities of carbon dioxide are absorbed from the surrounding air and consumed by the chloroplasts. It is a reasonable supposition, therefore, that in Exps. I and II, which were carried out in ordinary air, much or all of the respiratory and absorbed carbon dioxide was consumed in photosynthesis and little or none was available at centres of acid synthesis in the illuminated *Bryophyllum* leaves. Accordingly, little or no acid could be produced by leaves under these conditions even though glycolysis proceeded at a high rate.

We think that CO_2 supply may be the limiting factor in the rate of acid production in the light as long as photosynthesizing chloroplasts are not saturated with this gas. Clearly, in order to verify this postulate, it must be determined whether the rate of acid production may be augmented by enriching the atmosphere around the leaves with carbon dioxide beyond the capacity of illuminated chloroplasts to consume the gas in photosynthesis. thereby securing a steady supply of this essential metabolite to the centres of acid synthesis. The results of these crucial experiments will be reported and discussed in § III.

Light deacidification means that the rate of acid consumption exceeds that of acid production. Under certain conditions (e.g. low light intensity, high CO_2 concentration) considerable amounts of acid might be produced during light deacidification. For the present in making calculations (but see p. 433) we shall assume that at the high light intensities and summer temperatures used in all our experiments the rate of acid production in air fell virtually to zero, i.e. in any experiment acid consumption was equal to the measured loss in titratable acid in the light.

Accordingly, in T.A.N. units, the consumption of acid in Exp. I was 78 in 12 hr. of illumination, and in Exp. II it was 62 during the first 10 hr. and 47 during the second 10 hr. period of illumination. In Exp. II, temperature and light intensity were comparable during the two periods, and the fall in the rate of consumption during the second period may well relate to the lower acid concentration at the beginning of this period. Evidently if, contrary to our assumption, acid was actually produced in these experiments, the rate of acid consumption would have been higher than the rate of light deacidification. Deacidification also eventually occurs in leaves kept continuously in the dark. If the centres of acid consumption that operate in the dark are the same as those which bring about light deacidification (see Thomas, 1949), they, like the acid-synthesizing centres, will be potentially active in *Bryophyllum* leaves under all conditions, in the light or in the dark, and in air or in atmospheres enriched with carbon dioxide. An accumulation of acid (i.e. an increase in the T.A.N.) means that the rate of production exceeds that of consumption. The rate of accumulation of acid (V_a) in the dark will evidently be the difference between the rate of production (V_p) and the rate of consumption (V_c):

$$V_a = V_p - V_c.$$

In the second phase of Exp. II the average value of V_a, in terms of the T.A.N., was 40 in 12 hr. Evidently, the actual average rate of synthesis (V_p) of acid exceeded this value by an amount equal to the average rate of consumption (V_c). On the assumption that the potential rate of consumption is independent of light intensity and of CO_2 supply we can make a rough calculation of V_c from the data obtained in Exp. II. The third phase of the experiment was one of light deacidification in air (consumption without significant production, as we have argued above). During this phase the T.A.N. decreased by 47 in 10 hr., i.e. at a rate equivalent to about 56 in 12 hr. The average accumulation of acid substrate available for consumption during the previous period of dark acidification happened, by chance, to have been about the same as that during this final phase of light deacidification. It is possibly admissible to state, therefore, that the gross amount in T.A.N. units of acid produced during the dark period of 12 hr. was, very approximately, 96. Of this amount, 56 units were consumed. The net gain was 40.

What we have attempted is no more than a preliminary analysis of a complex problem, viz. the evaluation of V_p in the dark. Large-scale experiments in which courses of dark acidification, dark deacidification and light deacidification are simultaneously measured on comparable samples of leaves will have to be performed before a significant V_c value corresponding to a V_a value at any instant of time can be evaluated. At present V_a is the only magnitude we evaluate with certainty. This value is measured with great ease.

III. THE AUGMENTATION OF ACID PRODUCTION BY ENRICHING WITH CO_2 THE ATMOSPHERE AROUND ILLUMINATED *BRYOPHYLLUM* LEAVES

A. *Theoretical*

We have assumed that V_p was zero during the opening and final phases of Exp. II because, owing to photosynthesis, the CO_2 supply to centres of acid synthesis failed completely. According to this assumption the rate of loss of accumulated acid (V_l), i.e. that of light deacidification, was equal to V_c. Obviously if failure was not complete we should have to write

$$V_l = V_c - V_p.$$

Although V_p may be virtually zero in illuminated leaves surrounded with air, it is clearly arguable that it would become positive were the concentration of CO_2 around the leaves to be increased. Indeed, we should have lost confidence in the hypothesis which has guided this research had we failed to retard light deacidification by feeding *Bryophyllum* leaves with carbon dioxide. If acid consumption is not directly influenced by light, or by moderate CO_2 concentrations over short periods, retarded light deacidification would provide evidence of an enhanced rate of acid production. There was a clear possibility that it might be increased sufficiently for V_p to exceed V_c. Acid would then accumulate in the light, and the phenomenon, which we shall describe as light acidification, would be demonstrated.

The plan in most of the experiments to test these possibilities was to measure changes in the value of the T.A.N. of leaves from comparable samples, some of which were illuminated in an air stream and the remainder in gas streams containing supernormal but not narcotizing CO_2 concentrations, and to measure the further changes in these values when the leaves previously supplied with air were given gas and those previously supplied with gas were given air. Because we had only a limited number of *Bryophyllum* plants at our disposal we were forced, in most of our experiments, to make analyses at any one time on single samples of one or two leaves. The sampling error must have been high. Nevertheless, the trends of results obtained were similar in most of the experiments and were broadly in accord with the predictions made on the basis of the hypothesis. Accordingly, we now feel assured confidence in its validity.

B. *Experimental*

Exp. III (18 May). Twelve leaves of similar size were removed from three plants which had been darkened in the greenhouse overnight. Two of the leaves were used for the determination of an initial T.A.N. value (160) for the sample. Five of these very acid leaves were placed with their petioles in water in each of two vessels, through one (A) of which a stream of 8% CO_2+92% air was driven, whilst air was drawn through the other (B). Both were strongly illuminated and to the same extent. Single leaves were removed at the times stated in Tables 1 *a* and 1 *b* and their T.A.N.'s measured.

Table 1 *a*. *Retardation of light deacidification by carbon dioxide* (*Exp. III*)

Hours of illumination after dark period	T.A.N.	
	Leaves placed in 8% CO_2 (vessel A)	Leaves placed in air (vessel B)
0	160	160
5	146	48
7	146	52
23	132	20

The results recorded in Table 1 *a* provide clear evidence of retardation by carbon dioxide of light deacidification. Sampling errors may be too big for precise quantitative statements to be made; but, on the basis of these results, we can say that during 23 hr. in air the average T.A.N. fell about 90%, while in 8% CO_2 the fall was only about 20%. Carbon dioxide abundantly in excess of that consumed by the chloroplasts must have been available at centres of acid synthesis. We attribute the difference of 70% to the absorption and metabolic consumption of carbon dioxide by the detached leaves in vessel A in the synthesis of acid. The conclusion drawn is that, although light deacidification continued for longer than 7 hr. in the leaves in both vessels A and B, acid was synthesized by the leaves in vessel A in spite of the high light intensity. This may have been the first time that an experiment expressly designed to provide evidence that CO_2 feeding can induce acid production at high light intensities was successful in its object. Earlier experiments which gave results comparable with ours (see Thomas, 1949, p. 442) were performed with other objects in view. The general conclusion drawn from them was that carbon dioxide, by retarding consumption, retarded light deacidification.

Since the initial average T.A.N. of leaves in vessels A and B was about the same it may be permissible to calculate average values of V_c, V_t and V_p for the opening 5 hr. Assuming that 8% CO_2 does not affect the rate of acid consumption, V_c for leaves in both vessels A and B was 112. For the leaves in vessel A, V_t was 14. Hence V_p was 98. If it is allowed that acid production by leaves in vessel B was virtually zero, it follows that, by augmenting to 8% the CO_2 concentration in the atmosphere, and thereby increasing greatly the rate of supply of CO_2 to centres of acid synthesis, we succeeded in causing a synthesis by illuminated leaves in 5 hr. of acid equivalent in amount to a T.A.N. of 98.

If acid consumption for an invariable enzyme system is governed exclusively by acid concentration the maintenance of a high T.A.N. in the leaves in vessel A between 5 and 23 hr., and therefore of a high rate of acid consumption, suggests that acid production remained at a high level throughout this period. Indeed, if all our assumptions are justified, acid consumption over the whole 23 hr. period would not have been far short of 400 T.A.N. units. Deacidification was only 28 units. Accordingly, total synthesis may have been more than 350 units.

During the last 18 hr. the average rate of fall in the T.A.N. for leaves in vessel B was much slower than during the opening 5 hr. This decline can be attributed to the fall in the concentration of consumable acid.

The results of the second part of this experiment give support to the conclusions drawn above. After exposure for 7 hr. under the conditions described in Table 1*a*, two leaves were transferred from vessel A to vessel B, and two from B to A. Sixteen hours later the T.A.N.'s of these leaves were determined.

Table 1*b*. (*Continuation of Table* 1*a*)

Leaves transferred after 7 hr.	T.A.N. at 7 hr. (see Table 1*a*)	T.A.N. after 23 hr. of illumination
From A to B (air)	146	32
From B to A (8% CO_2)	52	84

We see that upon stopping the supply of CO_2 to leaves which were deacidifying very slowly in 8% CO_2, deacidification was accelerated to reach a rate comparable with that shown by illuminated leaves kept continuously in air. The T.A.N. fell by 114 in 16 hr. In our view acid synthesis stopped for want of the essential metabolite, carbon dioxide, and uncompensated consumption of acid prevailed.

On the other hand, the increase in the T.A.N. from 52 to 84 during 16 hr. for leaves transferred from air to 8% CO_2, if it is significant, provided evidence of the actual synthesis of acid in the light at a rate faster than the rate of consumption. This may have been the first successful induction of light acidification in an experiment designed for the purpose of demonstrating this phenomenon. Better evidence was obtained in Exp. IV.

The amount of acid synthesized during the last 16 hr. exceeded the amount of acid which accumulated in the light by the amount of acid consumed. We take the fall in the T.A.N. from 52 to 20 in air (Table 1*a*) as giving a rough measure (32), admittedly too low in theory, of the consumption, while the T.A.N. of the accumulated acid rose from 52 to 84 in 8% CO_2. For a light acidification value of 32, the minimum synthesis was, therefore, 64.

Exp. IV (1 June). Sixteen leaves, having an average T.A.N. of 28, were picked at noon on a dull day. Three were placed in air in the dark, and the remainder illuminated, some in air and the rest in an atmosphere containing 4% CO_2. T.A.N.'s were measured at the times stated in Table 2. In the experiments on the illuminated leaves, the gas streams were interchanged after 23 hr.

Table 2. *Influence of* CO_2 *in (a) retarding light deacidification and (b) promoting light acidification*

Hours from start	T.A.N.'s		
	Light		Dark
	4% CO_2	Air	Air
0	28	28	28
6	—	—	33
21	35	6	—
23	25	3	22
	Gas streams interchanged at 23 hr.		
	Air for 4% CO_2	4% CO_2 for air	
27	3	18	—
32	0	20	—
45	1	23	—

The results provide indisputable evidence of light deacidification in air before and after the interchange of gas streams. The fall in the T.A.N. from 25 to 3 during the 4 hr. after the change from 4% CO_2 to air indicates an average rate of acid consumption of, very approximately, 5 units per hour when the initial T.A.N. of the leaves ranged from about 20 to 35. During the first 23 hr. the amounts of previously accumulated acid did not change appreciably either in the leaves in air in the dark or in the leaves illuminated in 4% CO_2. If the arguments put forward on p. 426 are accepted, we may conclude that in both of these experiments acid synthesis occurred at the same rate as acid consumption, viz. at an average of approximately 5 units per hour.

Special interest attaches to the increase in the T.A.N. from 3 to 18, whilst leaves were exposed for 4 hr. to 4% CO_2 after they had consumed most of their accumulated acid during the preceding 23 hr. of illumination in air. This increase of 15 units provides better evidence of the phenomenon of light acidification than did the increase reported on p. 427. The actual synthesis of acid would be greater than 15 units, by an amount equal to the acid consumed during this period of 4 hr. Since the final T.A.N. was not high the rate of consumption must have been relatively fast.

Exp. V (mid-June). Ten leaves, picked during the day, were illuminated in the laboratory overnight. Analysis of two of the leaves on the following morning gave a T.A.N. of 36. Four of the remaining leaves were placed in light in 10% CO_2, i.e. in a higher concentration than we used in Exp. IV, and the other four in air. After illumination for 7 hr. (which, as the results of Exps. III and IV have shown, is a sufficient period for the detection of such marked effects, as may occur) the T.A.N. of the leaves in air had decreased to 19, while that of the leaves in 10% CO_2 had increased to 58. Expressed as T.A.N.'s, light acidification in the CO_2-enriched atmosphere was 22, while consumption was 17; hence production was 39. Evidently in CO_2 concentrations up to 10%, over a period of

7 hr., the promoting effect on acid synthesis of the increased active mass of this metabolite may be greater than any narcotizing influence it may exert, at this concentration, on enzymes taking part in the synthesis.

Exp. VI (15 July). When this experiment was done, suitable leaves were in very short supply. Now the leaves of *Bryophyllum* occur in pairs, and the sizes of opposite leaves are generally similar. To reduce sampling error as far as possible we cut off pairs of leaves, and placed one leaf from each pair in the mixture containing CO_2 and the other one in air. In each measurement we compared the T.A.N. of a leaf with that of its partner cut from the same node. Such procedure will be described as a leaf-pair experiment.

In Exp. VI two pairs (A and B) of leaves were illuminated for 6 hr. One leaf from each pair was then placed in 4·9% CO_2 and the other two leaves were left in air. Since, for obvious reasons, we do not know the average original T.A.N. of either leaf pair, we cannot calculate accumulation, consumption and production. Although we can be tolerably certain that in each pair some light deacidification occurred in the leaf illuminated in air, we cannot infer that light acidification took place in either of the two leaves illuminated in 4·9% CO_2. The main effect of carbon dioxide may have been to retard light deacidification.

Table 3. *Final T.A.N.'s of single leaves after 13½ hr. (see text, Exp. VI)*

	Leaf from air	Leaf from 4·9% CO_2
Leaf from pair A	18	77
Leaf from pair B	24	76

Evidently, however, the results recorded in Table 3 indicate that very marked differences relating to CO_2 supply may be obtained by experiments on single leaves provided these are taken from a single node. The results recorded in the vertical columns in Table 4 suggest that it is exceptional for leaves from dissimilar pairs, which have been kept under identical conditions, to give values so closely similar as are those recorded in Table 3.

Exp. VII (19 July). In another leaf-pair experiment, eight pairs were illuminated for 7 hr. One leaf from each pair was then placed in 5% CO_2 plus 95% oxygen and the other leaf in air. The leaves were analysed singly after further strong illumination for the periods stated in Table 4. After 21 hr. the gas streams were interchanged.

The horizontally placed numbers are for leaves from a single pair. Up to 21 hr. these numbers show consistently that T.A.N.'s are related to the presence in the atmosphere of carbon dioxide at an augmented concentration. It would be unwise to draw conclusions from the horizontally placed numbers for the two leaf pairs analysed 2 hr. after the interchange of gas streams. We think that the leaves previously in air were acidifying during this period of exposure to 5% CO_2, while those previously in 5% CO_2 were deacidifying in air. The differences in the values of the horizontally placed numbers for 25½ hr. are in accord with this view.

The vertically placed numbers show that for leaves from different pairs the sampling errors can be considerable. Although this experiment was not designed for the purpose, it may be justifiable, however, to calculate from these numbers the average values for groups of four leaves from different pairs. Before the interchange of gases that group of leaves in 5% CO_2 which was analysed had an average T.A.N. of 75. In the other group, which had been kept under identical conditions but was transferred from the 5% CO_2

to air, the average T.A.N. fell to 34 in a few hours. This fall may well be significant and attributable to normal light deacidification in air. Of more interest to our hypothesis is the rise of the average T.A.N. from 15 to 40 brought about in a few hours by substituting a gas stream containing 5% CO_2 for air. If significant, this rise indicates that, promoted by the additional carbon dioxide, acid production exceeded acid consumption. Collective consideration of the results of Exp. VII therefore provides support for the conclusion drawn from the results of Exps. III and IV, viz. that CO_2 feeding may not only retard light deacidification but may occasion light acidification.

Table 4. *Confirmatory evidence of the influence of* CO_2 *on acid metabolism in the light* (*Exp. VII*)

Duration of illumination in hours from start	T.A.N.'s of single leaves from a pair	
	Leaf in air	Other leaf of pair in 5% CO_2
18	31	73
21	11	113
21	9	61
21	9	52
	Gas streams interchanged	
	Leaf in air up to 21 hr. now in 5% CO_2	Other leaf of pair in 5% CO_2 up to 21 hr. now in air
23	68	91
23	20	16
25½	37	6
25½	36	21

Table 5*a*. *T.A.N.'s in Exp. VIII* (*see text*)

	Time in hours				
	3	4	5	21	22
Leaves in air	66	16	19	14	—
Leaves in 4% CO_2	91	87	70	53	93

Table 5*b*. *Fall in the T.A.N. from* 93 *at* 22 *hr.* (*see Table* 5*a*) *after substitution of air for* 4% CO_2

Time in hours from beginning of Exp. VIII	23	23½	24	26	28	28
T.A.N.	26	26	28	12	15	11

Exp. VIII (29 August). This experiment, which was not a leaf-pair experiment, was performed in the late summer on eighteen leaves of about the same age and size taken from different plants. Seven of the leaves were placed in air and the remainder in a gas mixture containing 4% CO_2. The T.A.N.'s of leaves were measured after illumination for the times stated in Table 5*a*.

Evidently, whereas in air the rate of consumption of acid greatly exceeded that of production during the first 4 hr., these rates throughout the 22 hr. were not very different when the illuminated leaves were supplied with 4% CO_2.

At 22 hr. air was substituted for the 4% CO_2 around the six remaining leaves, which, after further illumination, were analysed singly at the times stated in Table 5*b*.

The results recorded in Table 5*b* again provide clear evidence that illuminated leaves whose T.A.N. values have been maintained, or even enhanced, by supplying them with additional carbon dioxide (Table 5*a*), retain the power of consuming acid in the light. Indeed, the fall in T.A.N. from 93 to 26 in the first hour after the substitution of air for 4% CO_2 indicates a rate of consumption of acid comparable with that which occurred in air during the opening 4 hr. of this experiment.

Discussion

In Exps. III–VIII inclusive we have confirmed the findings of earlier workers (see Thomas, 1949, p. 442) that light deacidification is retarded by CO_2 feeding. We fed leaves with gas mixtures containing not more than 10% CO_2. In the earlier experiments higher concentrations were used. Furthermore, because we examined the effects of interchanging gas streams at certain stages of our experiments we have obtained unambiguous evidence that CO_2 concentration is one of the variables affecting light deacidification and acidification.

Unlike the earlier workers we attribute the retardation, not to the narcotic influence exerted by carbon dioxide on acid consumption, but to the promotion of acid production by the metabolic fixation of this gas in the Wood & Werkman reaction. In Exps. II, IV and VII evidence was obtained that production was promoted sufficiently for it to exceed consumption with resulting light acidification. This phenomenon had been observed before (see Thomas, 1949, p. 443), but the problem of explaining it proved perplexing. Clearly, a simple explanation may now be given if it is allowed that carbon dioxide is metabolically fixed in acid synthesis.

In illuminated leaves which are photosynthesizing rapidly in air the CO_2 concentration available for acid synthesis may approach zero. We postulate that acid consumption continues under all normal conditions and at potential rates which are independent of light intensity and such CO_2 concentrations as we have used. Evidently with the maintenance of a high potential rate of consumption and with a production rate that falls progressively as carbon dioxide is removed by photosynthesis from cell sap and intercellular spaces, accumulated acid will disappear. Provided the power of acid consumption is retained by leaves, light deacidification appears to be the inevitable result of vigorous photosynthesis in air.

Whether light deacidification, no change in acid concentration or light acidification occurs in atmospheres enriched with carbon dioxide will depend upon the rate of CO_2 supply to the centres of acid synthesis. Clearly this rate will be partly determined by the external CO_2 concentration and the relative vigour of photosynthesis. In some of our experiments (see results recorded in Table 1*a* and Table 2 for the opening 23 hr.) there must have been considerable acid production in leaves exposed to the atmosphere containing extra carbon dioxide, but the rate of production did not reach that of consumption. As the net result, therefore, light deacidification occurred, but at a rate greatly retarded relative to that of this process in the leaves in air. During certain phases of other experiments (see results recorded in Table 1*b*, in Table 2 for the period 23 hr., and Table 4 for the period after 21 hr.) the extra CO_2 so enhanced acid production that its rate exceeded that of acid consumption. The net result, therefore, was accumulation of acid in the light. This demonstration of light acidification afforded evidence that those events relating to acid metabolism which occur in the light in green leaves are qualitatively the same as those which take place in the dark. Light deacidification is not the inevitable result of a qualitative change induced by the direct action of light on acid metabolism. The events in the light and dark differ quantitatively because carbon dioxide is an essential metabolite for acid synthesis. The influence of light on acid metabolism is indirect, since it operates by controlling the CO_2 supply to centres of acid synthesis which maintain their potential powers unimpaired throughout a period of illumination.

There is no valid reason that we know for supposing that acid production stops completely even in leaves photosynthesizing at their maximum vigour in ordinary air. There will be competition between chloroplasts and centres of acid synthesis for respiratory carbon dioxide. The rate of supply of this gas to these centres will be very slow in comparison with this rate in darkened leaves, but does not necessarily fall to zero. We consider, therefore, that we have succeeded by CO_2 feeding, not in inducing the occurrence of a new process, but in accelerating the rate of a process already in operation, albeit very slowly, in the light.

The retardation of light deacidification and promotion of light acidification, which we brought about by CO_2 feeding, provided substantial support for the hypothesis which has guided this research. Evidently, however, assured confidence in the truth of the hypothesis could not be felt until evidence had been obtained that the carbon dioxide used to enrich artificial atmospheres is absorbed and consumed while the synthesis of acid is in progress. In 1946 leaves of *Bryophyllum* were in short supply, and the season of diurnal fluctuations was well advanced when this stage of the research was reached. We decided, therefore, to seek for evidence about CO_2 consumption in relation to acidification by carrying out experiments on darkened leaves. If it is allowed that the same mechanism is at work in the synthesis of acid in the light and in the dark, it follows that proof of CO_2 consumption during dark acidification is proof of CO_2 consumption during acid production in the light.

IV. GASEOUS EXCHANGE AND FLUCTUATION IN RESPIRATORY QUOTIENTS DURING ACIDIFICATION IN THE DARK IN AIR AND IN ATMOSPHERES ENRICHED IN CO_2

A. *Theoretical*

We have given reasons above for supposing that when *Bryophyllum* leaves are transferred from the light to the dark the only qualitative change which affects acid metabolism is that photosynthesis stops. Respiration and the production and consumption of malic acid continue. The processes of respiration and acid production may be summarized by the overall equations (i) and (ii) respectively. Equation (ii) formulates concisely the hypothesis (see Thomas, 1947, 1949) that the carbon contained in acids of the malic acid group is derived from carbon coming from carbohydrate and carbon dioxide (mainly respiratory), and that oxygen absorption is not directly involved in acid synthesis:

$$C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O, \tag{i}$$

$$C_6H_{12}O_6 + 2CO_2 = 2C_4H_6O_5. \tag{ii}$$

In the present investigation we obtained little evidence bearing on the problem of the consumption of titratable acid. Among the possible fates of malic acid are oxidation and oxidative anabolism. Possible overall equations for these processes may be written; (iii) for complete oxidation and (iv) for oxidative anabolism:

$$C_4H_6O_5 + 3O_2 = 4CO_2 + 3H_2O, \tag{iii}$$

$$2C_4H_6O_5 = C_6HO_6 + 2CO_2. \tag{iv}$$

The experiments to be described in the remainder of this paper have been performed mainly over periods in which production has greatly exceeded consumption, i.e. during vigorous dark acidification. Gaseous exchanges will thus have been predominantly those summarized in (i) and (ii). We realize, however, that the processes summarized in either (iii) or (iv), or in both of these equations, would occur simultaneously with processes (i) and (ii). For reasons given by Thomas (1949, p. 432) we shall assume for the present that consumption is mainly by equation (iv), involving no oxygen uptake.

Equations (i) and (ii) show clearly, as Thomas (1947, 1949) has already pointed out, that during dark acidification CO_2 production and CO_2 output will have different magnitudes. The carbon dioxide which is produced in respiratory oxidation (equation (i)) has at least two fates: (*a*) escape from the leaf to give a CO_2 output which is easily measured, and (*b*) metabolic consumption in a reaction or reactions of the Wood & Werkman type leading to the synthesis of vegetable acids (equation (ii)). On the basis of certain assumptions, this consumption can be calculated (see p. 443). Evidently the rate of escape will be determined by the relative rates of respiration and of acid synthesis by the leaves. When synthesis is relatively active, as it unquestionably is during acidification, since it then exceeds acid consumption, we may conclude that the CO_2 output will be relatively low. This conclusion is supported by experimental records of an inverse relation between rates of acidification and of CO_2 output (see Thomas, 1949, p. 434).

A fall in CO_2 output owing to the occurrence of the Wood & Werkman reaction will also explain why the values of respiratory quotients are low when plants showing Crassulacean acid metabolism are transferred from the light to the dark. The classical hypothesis was that these low R.Q.'s result from the simultaneous consumption of sugars in normal aerobic respiration (equation (i)) and by incomplete oxidation, with the absorption of oxygen but without the evolution of CO_2, according to equation (v):

$$2C_6H_{12}O_6+3O_2=3C_4H_6O_5+3H_2O. \tag{v}$$

It was thought that when R.Q.'s were relatively high, normal respiration was the more vigorous process; and that when they were low acid formation was dominant. Clearly it must be inferred that normal respiration stops when the R.Q. is zero. Oxygen uptake must then relate exclusively to acid production. We know of no quantitative results which would support this conclusion.

In the present paper we shall pay no regard to the possible occurrence of reaction (v). We shall argue from the assumptions (see Thomas, 1949, p. 432) that net oxygen uptake exclusively relates to normal respiration and that fluctuations in R.Q.'s during acidification can be explained in terms of equations (i), (ii) and (iv). When dark acidification is intense, a considerable fraction or even all of the respiratory carbon dioxide (equation (i)) will be consumed in acid synthesis (equation (ii)). Oxygen uptake will not be affected. Associated with respiration, it will continue as if acid synthesis were not occurring. Clearly, therefore, respiratory quotients will fall progressively and may reach zero as dark acidification intensifies, and will rise again when the rate of acid synthesis decreases. It has been argued (Thomas, 1949, p. 432) that if consumption is governed by equation (iv), the R.Q. will be unity when, after prolonged darkening, the rate of acid consumption is equal to that of acid production. Furthermore, when consumption is greater than production, i.e. during dark deacidification, the R.Q. should then rise above unity. Experimental results obtained by Astruč and by Bennet-Clark have already been cited (Thomas, 1949, p. 432) to show that it does.

We shall adhere to the established custom of evaluating the respiratory quotients of a plant organ from the volumes of carbon dioxide actually emitted by the plant into* and of oxygen taken in from the outside atmosphere, whether this is air or a gas mixture rich in carbon dioxide, without regard to the number and nature of the metabolic processes which involve the consumption or production of these gases. During the first flush of acid accumulation in the dark gaseous exchanges are largely determined by two reactions only, viz. those summarized by equations (i) and (ii). As a rule reaction (iv) need not be taken into account until dark acidification begins to slow down to a marked degree. In the present paper interest is centred mainly on events occurring prior to this retardation.

We hold that the rate of CO_2 output is a measure of the normal aerobic respiration of sugar only when the rate of acid consumption is equal to that of acid production. We shall assume that, at all stages of our experiments, aerobic respiration may be evaluated unambiguously from measurements of the rate of oxygen uptake. For the oxidation of sugar, considered as a reaction proceeding independently of acid metabolism, the respiratory quotient is unity. We can, therefore, calculate the rate of CO_2 production in normal sugar respiration, either in air or in gas mixtures containing carbon dioxide, from the results of our measurements of the rate of oxygen uptake. Evidently when the R.Q. of *Bryophyllum* leaves is less than unity, i.e. when acid accumulation goes on simultaneously with sugar oxidation, we shall expect to find that the calculated value of CO_2 production will be greater than the measured value of CO_2 output. Indeed, the difference between these values will give us the value of the net CO_2 metabolically fixed in the acid which has accumulated.

As this relation implies all the arguments so far put forward in this paper, and is, indeed, a succinct summary of them, we express it again as a verbal equation:

CO_2 fixed in accumulated acid = CO_2 production in sugar oxidation (calculated from measured oxygen uptake) − CO_2 output (measured by experiment).

Increases in T.A.N.'s give us the actual amounts of acid which accumulate during a period of dark acidification. Accordingly, we have laid the theoretical foundations for calculating the ratios of CO_2 fixation to acid accumulation over a period. Evidently if, in gas mixtures with augmented CO_2 concentrations, there is a simultaneous absorption of oxygen and CO_2, the equation will read

CO_2 fixed in accumulated acid = CO_2 produced in sugar oxidation (calculated from measured oxygen absorption) + CO_2 absorbed (measured by experiment).

Examples will be given in the discussion of the results obtained in Exp. XII.

We do not refer to carbon dioxide fixed in the synthesis or production of acid, but in acid accumulation, because of our assumption that acid is being consumed continuously. If equation (iv) represents consumption, CO_2 fixation in synthesis will obviously be greater than the CO_2 fixation corresponding to acid accumulation by an amount equal to the CO_2 produced during acid consumption. We shall have to wait until more definite information is available about acid consumption before attempting to evaluate the gross amount of carbon dioxide fixed by leaves in the dark. Theoretically, if the power to synthesize acid is always retained, such fixation will occur when they are undergoing dark deacidification and not only during dark acidification.

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* In Exps. X and XI, performed in atmospheres enriched in carbon dioxide, we have demonstrated the simultaneous absorption of carbon dioxide and oxygen. Regarding absorption as negative emission, we represent respiratory quotients in such instances with a negative sign (see Tables 7, 8 and 9).

B. *Experimental*

Simple demonstration that the R.Q. *of* Bryophyllum *leaves, synthesizing acid in the dark, may approach or fall to zero*

As our stock of *Bryophyllum* was limited and time was pressing, we did not carry out quantitative experiments on fluctuations in the gaseous exchange of leaves, enclosed initially in ordinary air in vessels kept in the dark. By means of a simple experiment similar in type to one of de Saussure's (see Thomas, 1949, p. 436), we satisfied ourselves, however, that our *Bryophyllum* leaves, when accumulating acids in the dark in the mid-summer of 1946, would have shown low R.Q.'s had such experiments been performed.

Exp. IX. To make them capable of vigorous dark acidification later, detached leaves were illuminated for a period to increase the carbohydrate and reduce the acid content. Comparable samples of five leaves each were placed in the top part of each of two stoppered towers (A and B) which had water manometers as side-arm fittings. A few c.c. of 20% caustic potash were placed in the basal part of A, but no CO_2 absorbent was placed in B. Both respirometers were then put in the dark. If carbon dioxide produced by the aerobic respiration of carbohydrate had escaped, the level of water in the manometer attached to A would have risen steadily, and, at constant temperature, that in the manometer attached to B would have remained unchanged. In all the experiments we carried out, however, the levels of water rose at about the same rates in both of the manometers. In one experiment the rise was about 20 in. in 12 hr. The results recorded in Table 6 show that dark acidification was vigorous in these experiments.

Table 6. *Dark acidification of leaves in air* (*see text*)

Average initial T.A.N.'s	Average T.A.N.'s after 12 hr. in dark	
	Leaves in tower A with KOH	Leaves in tower B without KOH
7	32	38
—	26	42
5	38	47
0	82	60

Evidently during dark acidification in these experiments oxygen was absorbed at about the same rates in towers A and B, and little or no carbon dioxide was evolved by the leaves. The respiratory quotient was near or equal to zero.

We conclude that the respiratory carbon dioxide produced in equivalent amount to the oxygen absorbed was nearly completely or completely consumed in the synthesis of acid and that little or none escaped from the leaves either in tower A or tower B. If none escaped the caustic potash was non-functional. The differences in the average increases of the T.A.N.'s of the leaves in A and B were not greater than those which, in discussing earlier experiments, we have attributed to sampling errors. If sugar concentration is not the limiting factor, equal rates of acidification mean equal rates of CO_2 supply to centres of acid synthesis, assuming these centres to have the same potential activity in comparable samples of leaves. The complete consumption of respiratory carbon dioxide without any escape in either tower A or tower B would account for the similar and fairly fast rates of acid synthesis.

Had a fraction of this carbon dioxide escaped because consumption was not complete, the level of water in the manometer attached to tower B would have risen more slowly than that in the manometer attached to A. The evolved fraction would have combined with the caustic potash in A while it accumulated in B. Oxygen would have been absorbed at similar rates in A and B. We should have concluded that the R.Q. lay between zero and unity. According to the literature it often does lie between these values during dark acidification, but many of the values recorded are averages for periods in the dark longer than those in our experiments. It still remains to be determined whether the R.Q. of leaves invariably changes from zero or from values near zero during the first flush of dark acidification in air to unity when acid ceases to accumulate. Clearly such a rising R.Q. would give an average R.Q. of between zero and unity for the whole period. This average value would depend upon the duration of the period (see Thomas, 1949, p. 432).

Evidence that, during dark acidification in atmospheres enriched in carbon dioxide, CO_2 *consumption may occur simultaneously with absorption of oxygen, and so give negative values of respiratory quotients*

None of the experimental results so far recorded or discussed in this paper has provided indisputable evidence of the metabolic consumption of carbon dioxide during acid synthesis, but practically all of them have been successfully interpreted on the assumption that carbon dioxide is so consumed. We now come to reports of the crucial experiments which substantiate the hypothesis. The results of Exps. X, XI and XII may have provided the first clear evidence that carbon dioxide can be absorbed, simultaneously with oxygen, by green leaves in the dark over a period of several hours while acid is accumulating in them. Thomas (1949, p. 436) has called this absorption 'the de Saussure effect'. De Saussure observed the phenomenon in *Opuntia*, but the acid metabolism of this genus was not discovered until later.

The problem was attacked in a simple way. It was argued that if the measured R.Q. of *Bryophyllum* leaves surrounded by air were zero (Exp. IX), it might well be that in the dark the leaves could consume carbon dioxide from the external atmosphere in addition to their own respiratory carbon dioxide. The obvious way to determine whether or not the leaves possessed this power was to enclose them in an atmosphere enriched in carbon dioxide, but not at poisoning concentrations, and to find out if some or all of the carbon dioxide were absorbed. A decrease in the amount of carbon dioxide in this artificial atmosphere could have no other meaning than (*a*) solution in the leaf sap, (*b*) chemical combination with a leaf constituent, or (*c*) metabolic consumption. In the event, as we shall see, such a decrease occurred. Since there are good reasons for discarding explanations (*a*) and (*b*) as the main causes of this decrease, we think that we have obtained evidence of the metabolic consumption of carbon dioxide in the dark by *Bryophyllum* leaves which is quite as convincing as that which was obtained by the pioneer workers on the process of photosynthesis for the consumption of carbon dioxide in the light. Moreover, since this consumption invariably related to acid accumulation, we infer that it resulted from CO_2 fixation in a Wood & Werkman reaction or in another of similar type. Clearly this inference requires substantiation by biochemical experiments on *Bryophyllum*, like those which have been performed on bacteria, animal tissues and parsley roots (see Thomas, 1949, pp. 424–5).

Exp. X (16 August). A good crop of leaves was available for this experiment. They were gathered in the evening; and, as in Exp. IX, the leaves were illuminated in the laboratory overnight. In the morning three were analysed; their average T.A.N. was 47. The remaining leaves were enclosed in a vessel containing a gas mixture of 8·9% CO_2, 20·8% oxygen, and nitrogen. The vessel was placed in the dark. Samples of gas were withdrawn and analysed at the times stated in Table 7. After 23 hr. the gas mixture was replaced by air. Further gas analyses were made 1 and 2 days later. The T.A.N.'s of samples of leaves were determined at the time of the change-over from the gas mixture to air and at the end of the experiment.

Table 7. *Changes in the percentage gas composition by volume, caused by the combined effects of respiration and dark acidification, in* Bryophyllum *leaves, in atmospheres artificially enriched in carbon dioxide* (*Exp. X*)

Time from start in hours	T.A.N.	% CO_2 in gas mixture	% CO_2 output during successive periods	% oxygen in gas mixture	% oxygen absorption during successive periods	Average R.Q. during successive periods (see p. 424)
0	47	8·9		20·8		
			−1·2		0·9	−0·93
2	—	7·7		19·9		
			−0·4		0·5	−0·71
4	—	7·3		19·4		
			−1·2		1·7	−0·66
6	—	6·1		17·7		
			−0·6		2·1	−0·34
8	—	5·5		15·6		
			1·6		3·1	−0·46
23	130	7·1		12·5		
The gas mixture was then replaced by air						
23	130	0		20·8		
			2·0		2·3	0·79
47	—	2·0		18·5		
			1·5		1·2	1·27
71	109	3·5		17·3		

The results recorded in Table 7 show that during the opening 23 hr. of Exp. X, the T.A.N. of acid synthesized by the leaves in the gas mixture exceeded that of acid consumed by 83. The results obtained by Bennet-Clark (1933*b*) in his experiments on dark acidification in air by plants showing Crassulacean acid metabolism, and the results obtained in our Exp. XIII, which indicate that carbon dioxide hastens the attainment of the acid maximum, render it probable that in Exp. X most of the acid accumulated during the first half of the opening phase of 23 hr. Whilst the acid accumulated carbon dioxide was absorbed from the gas mixture surrounding the leaves. The percentage carbon dioxide in this atmosphere fell continuously, the total apparent fall was 3·4% in 8 hr. This experiment may have provided the first clear evidence that certain green plants can absorb carbon dioxide over a period of several hours in the dark whilst they are synthesizing acid. The actual CO_2 consumption while acid was accumulating was greater than the CO_2 absorption by an amount equivalent to the CO_2 produced in aerobic respiration (see p. 435). Oxygen absorption was continuous throughout the 8 hr., the total apparent absorption was 5·2 %. If the R.Q. for aerobic respiration is unity, the amount of respiratory carbon dioxide produced would have given an apparent output of 5·2 % had the carbon dioxide escaped into the atmosphere surrounding the leaves. As it did not escape, we infer that it was consumed in acid synthesis. Hence the total apparent fixation of carbon dioxide in the synthesis of such acid as accumulated during the opening 8 hr. was 8·6 %. The method of calculating the absolute amounts of oxygen absorption and of net CO_2 fixation will be described by Thomas & Ranson (see p. 424). If acid is continuously consumed with the evolution of CO_2, the total amount of carbon dioxide actually metabolized in the synthesis of acid would be greater than the net CO_2 fixation.

It was in this experiment that the phenomenon of the simultaneous absorption of carbon dioxide and oxygen by darkened leaves was rediscovered (see p. 437). In experimental records in this and subsequent papers negative values of R.Q.'s indicate its occurrence (see footnote, p. 435). We maintain that such negative values imply that leaves possess strong powers of fixing carbon dioxide in a Wood & Werkman reaction.

We note that at some time between 8 and 23 hr. in Exp. X, while there was still plenty of carbon dioxide in the external atmosphere, this simultaneous absorption with a negative R.Q. gave place to the normal gaseous exchange of CO_2 output and oxygen uptake. The positive R.Q. then obtained was much lower than unity. We conclude that only a fraction of the respiratory carbon dioxide escaped from the leaves, the remainder being used in the synthesis of such acid as accumulated during these last 15 hr. We shall not attempt a detailed analysis of the second phase of Exp. X. It appears that during these two days in air, starvation conditions were beginning to prevail. The behaviour of the leaves was similar to that which they might have been expected to show at this stage had they been kept in air in the dark throughout the experiment (see Bennet-Clark, 1933*b*). Dark deacidification may have commenced because production fell short of consumption. The limiting rate in the process of production would not be that of CO_2 supply but of the formation of pyruvic acid, i.e. the product of glycolysis which combines with carbon dioxide in the Wood & Werkman reaction.

Table 8. *Exp. XI*

Time from start in hours	T.A.N.	% CO_2 in gas mixture	% CO_2 output during successive periods	% oxygen in gas mixture	% oxygen absorption during successive periods	Average R.Q. during successive periods (see p. 424)
0	50	6·6		20·3		
			−1·0		0·8	−0·92
3	—	5·6		19·5		
			−3·3		0·8	−2·0
5	—	2·3		18·7		
			−1·6		0·6	−1·4
7	—	0·7		18·1		
			4·0		6·0	0·61
24	—	4·7		12·1		
			0·7		0·6	1·4
27	—	5·4		11·5		
			0·7		0·6	1·2
30	130	6·1		10·9		

Exp. XI (19 August). In Table 8 we record the results obtained in an experiment resembling the opening phase of 23 hr. in Exp. X. As the trend of the results is similar in the two experiments we shall make categorical statements about the results obtained in Exp. XI. Oxygen and carbon dioxide were simultaneously absorbed during the opening 7 hr. when most of the dark acidification responsible for the net increase in 30 hr. of 80 in the T.A.N. probably occurred. Accordingly, the R.Q. was negative over this period. The total apparent CO_2 fixation in the synthesis of such acid as accumulated in 7 hr. was 8·1 %.

At some time between 7 and 24 hr. respiratory carbon dioxide began to escape from the leaves, augmenting the external concentration of this gas, which had become greatly reduced during the opening 7 hr. Since the average R.Q. over these 17 hr. was 0·61, we infer that some acid accumulated during the period. It is possible that the R.Q.'s of 1·4 and 1·2 found for the last 6 hr. of the experiment mean that dark deacidification was in progress during this period. If it was, the maximum accumulation of acid, having a T.A.N. greater than 130, would have occurred at some time before the end of the experiment.

Exp. XII (26 August). In Exps. X and XI we found that carbon dioxide was mainly absorbed from the atmosphere enriched in this gas during the first few hours after darkening the leaves. We argued that this rapid absorption was correlated with rapid dark acidification. Exp. XII, which lasted 30 hr., was designed to get some information about such acidity changes as occurred simultaneously with changes in composition of the external atmosphere during the first 10 hr. as well as over the whole period.

After two fine days we cut *Bryophyllum* leaves and illuminated them overnight, presuming that the carbohydrate concentration would then be relatively high and the acidity relatively low. The leaves would thus have a relatively high potential power of accumulating acid, and, therefore, of fixing carbon dioxide. They were then placed in four gas jars, in numbers such as to ensure that the ratio *gas space/leaf volume* was not widely different from jar to jar:

Jar 1 had 61·0 g. of leaves in 890 c.c. gas space.
Jar 2 had 25·1 g. of leaves in 380 c.c. gas space.
Jar 3 had 16·5 g. of leaves in 210 c.c. gas space.
Jar 4 had 15·1 g. of leaves in 265 c.c. gas space.

Each jar was filled with a gas mixture containing 4·3 % CO_2 and 19·1 % oxygen, and placed in the dark. At the times recorded in Table 9 samples of gas from jar 1, the largest jar, were analysed.

The general trend of the results is similar to that of the results recorded in Tables 7 and 8 for our earlier experiments. From the records of negative R.Q.'s up to 9 hr., and from the low value of the R.Q. found immediately after, we conclude that acidification was relatively active during the first 10 hr. of this experiment. Actual measurements of acidity support this conclusion. We measured the average T.A.N.'s of a sample of leaves at the outset, and of all the leaves in each jar at the end of the experiment for which the jar had been used. The data obtained are recorded in Table 10.

In Fig. 1 we have plotted changes in gas composition calculated (see p. 424) from the data in Table 9 and the changes in T.A.N.'s recorded in Table 10. Admittedly the judgement used in drawing the curves has been somewhat subjective. Data for the first 4 hr. and for the period between 10 and 24 hr. would be required before the precise course of events could be confidently figured. Nevertheless, the curves serve the purpose of summarizing our general conclusions. (1) Acid accumulation progressed most rapidly during the first 10 hr. or so. (2) Correlated with this rapid accumulation, carbon dioxide was absorbed from the gas mixture, i.e. there was a negative output for a period lasting between 6 and 9 hr. During this period the R.Q. was negative. (3) Oxygen was absorbed continuously throughout the experiment. Were this absorption entirely attributable to the respiratory oxidation of carbohydrate, and had there been no acid accumulation, the curve for CO_2 output would have coincided with that of oxygen uptake, as it does approximately for ivy leaves placed in a gas mixture containing 5·9% CO_2 (see Fig. 2). We note that later in the experiment the curves may be nearly parallel for a period, indicating a phase in which the average R.Q. is close to unity. Clearly our data are not sufficiently numerous for us to state categorically that the acid concentration was maintained at a constant value while the R.Q. was unity. If it was so maintained, we may argue to an important conclusion about acid consumption (see Thomas, 1949, p. 432). An unchanging amount of accumulated acid means that the rates of production and consumption are equal. Hence, since carbon dioxide is absorbed in production, it must be liberated in consumption. Further discussion will be deferred until better evidence is recorded in a later paper in this series.

Table 9. *Changes in the percentage composition of gas by volume in jar* 1 (*Exp. XII, see text*)

Time from start in hours	% CO_2 in gas mixture	% CO_2 output in successive periods	% oxygen in gas mixture	% oxygen absorption in successive periods	Average R.Q. during successive periods (see p. 424)
0	4·3		19·1		
		−2·8		0·3	−2·8
4½	1·5		18·8		
		−1·1		0·8	−0·9
6½	0·4		18·0		
		−0·2		0·6	−0·3
9	0·2		17·4		
		0·3		0·8	0·3
10	0·5		16·6		
		3·9		4·3	0·9
24	4·4		12·3		
		1·2		0·6	2·4
27	5·6		11·7		
		0·8		1·2	0·6
30	6·4		10·5		

Table 10. *Changes in average* T.A.N.*'s during the course of Exp. XII* (*see text*)

Hours from start	Jar number	T.A.N.	% CO_2 in gas mixture	% oxygen in gas mixture
0	All jars	27	4·3	19·1
3	2	—	2·9	19·6
5	2	70	1·1	—
6	3	60	1·9	17·5
7	4	—	2·2	18·6
10	4	78	2·3	18·2
30	1	92	6·4	10·5

Beyond reasonable doubt normal sugar respiration was in progress when the R.Q. is unity. We hold that it occurred throughout the experiment at a rate represented by the curve for oxygen uptake drawn in Fig. 1. It would follow that the respiring cells in the leaves *produced* carbon dioxide at the same rate. Accordingly, when the R.Q.'s were negative this respiratory carbon dioxide, in addition to the carbon dioxide absorbed from the environment, must have been totally consumed in acid synthesis. When the R.Q.'s lay between zero and unity, only a fraction of the respiratory carbon dioxide was consumed; the remainder escaped from the leaves, giving a CO_2 output.

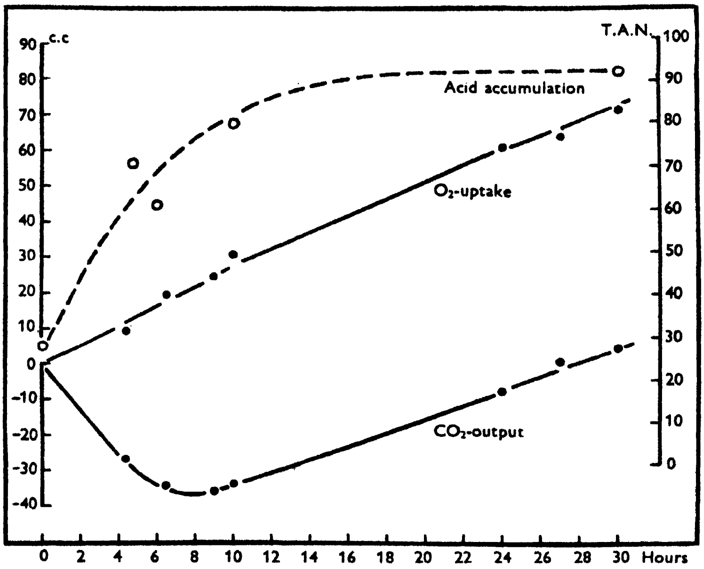

Fig. 1. Oxygen uptake and CO_2 output in c.c. by a single sample of 61 g. of *Bryophyllum* leaves, calculated from the data in Table 9, and acid accumulation measured in different samples (see Table 10), during the course of Exp. XII. At the start the gas mixture around the leaves contained 4·3 % CO_2 and 19·1 % oxygen.

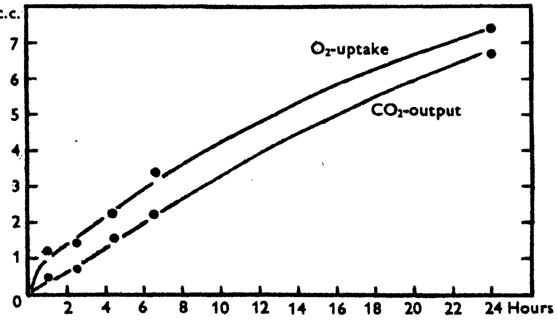

Fig. 2. Oxygen uptake and CO_2 output in c.c. by a sample of ivy leaves placed in a gas mixture containing 5·9 % CO_2 and 20·8 % oxygen. Ivy leaves do not show Crassulacean acid metabolism. The difference in the shapes of charts for CO_2 output in Figs. 1 and 2 provides evidence that little or none of the CO_2 absorption in Exp. XII is attributable to solution in cell sap (see summarized conclusions, para. 8).

If the only physical, chemical or metabolic changes affecting gaseous exchanges and the production and consumption of accumulated titratable acid were (*a*) malic acid production with CO_2 fixation (equation (ii), p. 433) but not involving oxygen absorption, (*b*) normal aerobic respiration with an R.Q. of unity (equation (i), p. 433), and (*c*) CO_2 production in acid consumption (equation (iv), p. 433), the weight of malic acid actually found, by titration, to accumulate should be equal to the weight of malic acid calculated from equation (ii) as equivalent to net CO_2 consumption. Obviously, with such an equality, the ratio *acid accumulation/net* CO_2 *consumption* would be constant; we should then be prepared to affirm that our quantitative arguments had been proved to be valid and, of their kind, complete. With a constant ratio, but without such equality, our view would be that our arguments, though sound as far as they went, were not quite complete. A fluctuating ratio would compel us to admit, however, that processes such as solution of carbon dioxide in cell sap, or as gaseous exchanges and forms of acid metabolism other than those we have considered, would have to be studied before the problem on which we have been working could, within the limits set, be completely elucidated.

The critical magnitudes mentioned in the last paragraph can be calculated from data of the kind given in Table 10, since these record simultaneous changes in T.A.N. and in the composition of gas around leaves in single samples. During 6 hr. in jar 3 (gas space approximately 210 c.c.) the T.A.N. (see definition on p. 423) of 16·5 g. of leaves had increased by 33, while apparent decreases of 2·4% CO_2 and of 1·6% oxygen occurred simultaneously in the composition of the external atmosphere. The calculated actual decreases (see p. 424) are 5·24 c.c. of CO_2 and 5·19 c.c. of oxygen. Since 1 c.c. of N/10-sodium hydroxide is equivalent to 6·7 mg. malic acid, it follows that 36·5 mg. of this acid accumulated in 16·5 g. of leaves. We argue (see p. 435) that the volume of carbon dioxide consumed in bringing about this increase of 36·5 mg. in accumulated malic acid was 10·43 c.c., i.e. the sum of the volumes of carbon dioxide absorbed and of the CO_2 equivalent of the oxygen absorbed. At the temperature and pressure prevailing when the experiment was performed this volume corresponded to 20·4 mg. carbon dioxide. The results of these calculations are recorded in Table 11 with results obtained in similar calculations from other data given in Table 10.

Table 11. *Comparison of the weights of accumulated malic acid found experimentally* (*column* 5) *and calculated* (*column* 6) *on the basis of equation* (ii) *written on p.* 433, *from weights of* CO_2 *consumed*

(1) Hours	(2) Jar	(3) Weight of leaves (g.)	(4) Net CO_2 consumed by leaves (mg.)	(5) Malic acid accumulation (mg.)	(6) Acid accumulation / Net CO_2 consumption	(7) Malic acid equivalent to CO_2 consumed (mg.)
6	3	16·5	20·4	36·5	1·8	62
10	4	15·1	18·8	51·5	2·7	57
30	1	61·0	130·2	266	2·0	397

In later papers of this series without further introductory comment, the malic acid equivalent of the net CO_2 consumption will, whenever possible, be compared with the actual accumulation, and the ratio *acid accumulation/net* CO_2 *consumption* will be calculated. The inconclusive results recorded in Table 11 are sufficiently discussed in para. 8 of the summarized conclusions at the end of the present paper.

A preliminary consideration of the influence of external CO_2 *concentration on (a) the rate of dark acidification, and (b) the maximum value reached for the concentration of accumulated acid*

When carbon dioxide is absorbed in the dark by *Bryophyllum* leaves placed in gas mixtures enriched in this gas, it is a reasonable supposition that the rate of dark acidification in a comparable sample of leaves placed in air would be limited by the supply of respiratory carbon dioxide. Time allowed for only one experiment to test this supposition.

Exp. XIII (early September 1946). A batch of fairly large leaves was illuminated in the laboratory overnight. Three of the leaves were then analysed, giving a T.A.N. of 47. Four leaves were placed in each of four jars filled with a gas mixture containing initially 2·15% CO_2, and the rest of the leaves in a vessel open to the air. The jars and vessel were kept in the dark. At the times stated in Table 12 a jar was removed from the dark. The percentage carbon dioxide in the gas contained in it was determined and the T.A.N. of the leaves measured. At the same time measurements were made of the T.A.N. of two of the leaves exposed to air. This procedure was not completely satisfactory because the external CO_2 concentration in the gas mixture changed during the course of the experiment. Nevertheless, we succeeded in obtaining some evidence concerning the influence of CO_2 feeding on the initial rate of acid accumulation in the dark.

Table 12. *Exp. XIII. The influence of* CO_2 *concentrations on the rate and the maximum amount of acidification*

Time in hours from start	T.A.N.'s		% CO_2 in jars containing gas mixture
	Leaves continuously in air	Leaves in gas mixture	
0	47	47	2·15
2	40	96	1·86
2¾	51	84	1
5½	—	115	Nil
24	128	125	2·3

First we note that, as in Exp. XII, the most rapid phase of acidification in leaves placed in the gas mixture occurred while carbon dioxide was being absorbed from the external atmosphere. During the last 18½ hr. the increase in the T.A.N. was only 10, and what must have been a considerable fraction of the respiratory carbon dioxide was given off by the leaves and accumulated in the jar which contained them.

A feature of this experiment was that while the value of the T.A.N. for leaves in the gas mixture was doubled approximately during the opening 2¾ hr., when an external supply of carbon dioxide was available, the value of the T.A.N. for the leaves in air did not change appreciably. The increase of 77 in the T.A.N. during the last 22¼ hr. showed that the leaves had the power to accumulate acid in air. Indeed, the maximum value of the T.A.N. found for the leaves in air was approximately equal to that found for the leaves initially in the gas mixture. The data are too few for us to do more than draw the tentative conclusion that CO_2 feeding of *Bryophyllum* leaves in the dark may possibly accelerate the rate of acidification, but may not greatly alter the peak value of accumulated acid. This conclusion will be tested later by experiments in which leaves are made to accumulate acid when exposed to gas streams of constant composition, each containing different concentrations of carbon dioxide.

SUMMARIZED CONCLUSIONS

(1) When leaves of plants belonging to the genus *Bryophyllum* are showing the diurnal fluctuations in titratable acidity that characterize Crassulacean acid metabolism, light deacidification may be strongly retarded by enriching the atmosphere around the leaves with carbon dioxide. When ordinary air is substituted for the enriched atmosphere, the retarding influence is removed. Certain earlier workers who also had observed this retardation (see Thomas, 1949) attributed it to the inhibition by carbon dioxide of acid consumption. The interpretation given previously (Thomas, 1947, 1949) and in the present paper is that the predominant effect of carbon dioxide is to promote the Wood & Werkman reaction, possibly already in operation, although at a slow rate, and so to occasion vigorous acid production. Even if acid consumption is not inhibited, light deacidification (i.e. loss in light of accumulated titratable acid) would be retarded by the enhanced rate of acid production.

(2) The interpretation outlined in (1) will account for the occasional occurrence in our experiments and in those of earlier workers (see Thomas, 1949) of light acidification. When this effect is observed, we think that acid production has been enhanced to such an extent that it exceeds consumption. Our experiments were designed with the express purpose of showing that CO_2 feeding, by promoting the Wood & Werkman reaction, could bring about the accumulation of acids in illuminated leaves. They were crucial experiments to test a new hypothesis, and predictions were fulfilled.

(3) Mainly to emphasize the fact that acid accumulation is less than production, approximate evaluations have been made of production in air. These evaluations are based on the assumptions (*a*) that acid production in light is zero (this may not always be strictly true, see p. 433), and (*b*) that, for a given initial amount of acid substrate, the rate of acid consumption is the same in the light as in the dark. The total acid produced in the dark over a given period would then be the sum of the amount of acid which is measured as accumulating in the dark in a sample of leaves and of that which disappears in the same or a comparable sample over the same period in the light.

(4) The major discovery recorded in this paper is that when *Bryophyllum* leaves are placed in the dark in an atmosphere moderately enriched with carbon dioxide they absorb oxygen and carbon dioxide simultaneously as long as acids are accumulating in them, but no longer. De Saussure observed this simultaneous absorption of the two gases in *Opuntia*, but did not know that this genus has an acid metabolism. Later investigators either took no notice of de Saussure's observation or concluded (see Thomas, 1949, p. 437) that it had no bearing on acid metabolism. Aubert attributed the observation to the solution of carbon dioxide in the cell sap. In the next paper Thomas & Ranson hope to present conclusive evidence for *Bryophyllum* and other genera that the continued absorption is mainly, at least, a result of metabolism and not merely of solution. In the present paper a comparison is made between the behaviour of ivy leaves (Fig. 2), which do not show Crassulacean acid metabolism, and *Bryophyllum* leaves (Fig. 1) in gas mixtures of similar composition. The charts depict that carbon dioxide was unmistakably absorbed by *Bryophyllum* leaves during the opening seven or eight hours whilst acid metabolism was proceeding at a relatively fast rate. There was clearly no corresponding massive absorption by ivy leaves. The only suggestion that carbon dioxide might have dissolved in cell sap to a slight extent comes from the evidence that in the gas mixture the

respiratory quotient of the ivy leaves appeared to be less than unity. Since the gaseous exchange of ivy leaves in ordinary air was not measured this point, which is at present a minor one, cannot be regarded as settled. It is however clear that the R.Q. of ivy leaves was maintained throughout at a value not far from unity, indicating that gaseous exchange was predominantly determined by the normal aerobic respiration of carbohydrate. A large-leaved species of *Saxifraga* gave results similar to those obtained with ivy.

(5) In a simple experiment of the kind which might be performed in elementary classes it was demonstrated that, while *Bryophyllum* leaves were undergoing vigorous dark acidification for twelve hours in air, the value of the respiratory quotient was zero. It has already been argued (Thomas, 1949, p. 430) that R.Q.'s between zero and unity provide evidence that all or some of the carbon dioxide produced in normal respiration is consumed in the synthesis of acid and, therefore, does not escape. It is assumed that net oxygen uptake is unaffected by the occurrence of acid metabolism.

(6) The R.Q.'s are negative when carbon dioxide and oxygen are simultaneously absorbed from mixtures of these two gases. Wherever a negative R.Q. for a period is recorded in tables in the present paper, we infer that the Wood & Werkman reaction took place during that period. Our data are, however, too few for confident statements to be made about the stages of acid accumulation at which these negative R.Q.'s reach their minimum values and later rise through zero to unity. The results recorded in the tables merely show the general trend of fluctuations in the respiratory quotients.

(7) The shapes of the charts drawn in Fig. 1 are too conjectural for detailed arguments to be admissible. We maintain, however, that when acid accumulation is approaching its maximum value, gaseous exchange in *Bryophyllum* leaves is similar to that in ordinary (e.g. ivy) leaves, and is determined predominantly by the normal aerobic respiration of carbohydrate. Taking oxygen uptake as a measure of this respiration, we infer that, in all our experiments, it proceeded at a steady rate throughout the dark period.

(8) From measurements of oxygen uptake the corresponding amounts of carbon dioxide actually produced in aerobic respiration have been calculated. On adding to them the amounts of carbon dioxide absorbed from gas mixtures we have in three instances been able to evaluate the *net* amount of carbon dioxide fixed while a measured amount of acid accumulated, and, therefore, the ratios of these amounts. These ratios are not obviously constant. In only one of the three instances was there a reasonably close agreement between the actual accumulation and the amount of malic acid calculated as equivalent to carbon dioxide according to the overall equation

$$C_6H_{12}O_6 + 2CO_2 = 2C_4H_6O_5$$

which summarizes one part of the hypothesis (Thomas, 1947, 1949) that the experiments described in the present paper were designed to test. In two of the instances considerably more carbon dioxide was consumed than could be accounted for by this equation. It remains to be determined whether the discrepancy is owing to errors intrinsic in the analytical methods we have used, or to faulty or incomplete theoretical arguments.

(9) In using the word *net* in the last paragraph, we had in mind (*a*) the long-accepted view that acid consumption takes place simultaneously with acid production during dark acidification, and (*b*) the contention (see Thomas, 1949, p. 439) that carbon dioxide is produced when titratable acid disappears from cells. If in Exp. XII (see Fig. 1) the amount of accumulated acid was truly constant while the R.Q. was unity, this contention

would be supported. The three R.Q.'s greater than unity, which are recorded in Tables 8 and 9, may have been found during a phase of dark deacidification after the maximum accumulation of acid had been reached. These three values may be grouped with those obtained by Astruč and by Bennet-Clark (see Thomas, 1949, p. 432) as providing evidence that the net CO_2 turn-over at centres of acid metabolism leads to liberation of this gas when consumption exceeds production. The suggestion has been made (Thomas, 1949, p. 440) that consumption may possibly be summarized by the overall equation

$$2C_4H_6O_5 = C_6H_{12}O_6 + 2CO_2.$$

(10) All the results reported in the present paper support the main assertions made in the paper introducing this series, viz. (*a*) diurnal changes in acidity in Crassulacean acid metabolism relate to fluctuations of CO_2 concentration at centres of acid synthesis, (*b*) these fluctuations result from the consumption in photosynthesis by illuminated leaves of respiratory carbon dioxide, which, in contrast, is available in the dark for acid synthesis, and (*c*) whereas in acid production there is a fixation of carbon dioxide by the Wood & Werkman reaction, in acid consumption carbon dioxide is produced and, in the dark, liberated with respiratory CO_2 from the leaves.

REFERENCES

BEEVERS, H. (1947). Some effects of CO_2 on plant metabolism. Ph.D. Thesis, Durham University.

BENNET-CLARK, T. A. (1933*a*). The role of the organic acids in plant metabolism. Part I. *New Phytol.* **32**, 37.

BENNET-CLARK, T. A. (1933*b*). The role of the organic acids in plant metabolism. Part II. *New Phytol.* **32**, 128.

PUCHER, G. W., LEAVENWORTH, C. S., GINTER, W. D. & VICKERY, H. B. (1947*a*). Studies in the metabolism of Crassulacean plants: The effect upon the composition of *Bryophyllum calycinum* of the form in which nitrogen is supplied. *Plant Physiol.* **22**, 205.

PUCHER, G. W., LEAVENWORTH, C. S., GINTER, W. D. & VICKERY, H. B. (1947*b*). Studies in the metabolism of Crassulacean plants: The diurnal variation in organic acid and starch content of *Bryophyllum calycinum*. *Plant Physiol.* **22**, 360.

THOMAS, M. (1947). *Plant Physiology*. London.

THOMAS, M. (1949). CO_2 fixation and CO_2 liberation in Crassulacean acid metabolism. *New Phytol.* **48**, 390.

THOMAS, M. & RANSON, S. L. Further evidence of CO_2 fixation and new evidence of CO_2 liberation in plants showing Crassulacean acid metabolism. (In preparation.)

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MORPHOLOGY OF THE STAMINATE FLOWER OF BOTTLE GOURD, *LAGENARIA LEUCANTHA* (DUCH.) RUSBY

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(With 4 figures in the text)

The morphological nature of the staminate flower of Cucurbitaceae was a subject of long-standing discussion, and unfortunately its exact nature has not yet been clearly understood. Stamens in the Cucurbitaceae have been described usually as two and a half, or three, or five (see references). The difficulty of comprehension of the monothecal type of the anther unusual in flowering plants involved arguments for considering the double thecal stamens (Clarke, 1879) as complete and the odd one with one theca as a half-stamen—one theca in the latter case being considered not developed.

In order to determine the real significance of this interesting structure, I have undertaken a thorough morphological study of the staminate flowers of *Lagenaria leucantha* (Duch.) Rusby (*L. vulgaris* Ser.) along with other species. The plants were raised at the glasshouse of the Cambridge University Botanic Garden from seeds procured from India and other sources. Seeds were sown in late May or early June, and flowering started from the beginning of September. Serial microtome sections of the male flower buds of different ages were cut at 10–15 μ thickness. The materials were fixed and killed in Belling's modification of Navashin's fixative (Lee, 1937). Light green in equal parts of xylol and clove oil or fast green dissolved in clove oil and safranin dissolved in 50% alcohol were found suitable combination-stains for distinguishing lignified from cellulose tissues. Freehand sections of mature flower buds or flowers were employed whenever necessary. For determining the courses of vascular strands from the pedicel to the floral members thick longisections of flower buds or semi-open flowers were cut by razor, boiled in pure lactic acid for about half an hour and mounted in lactic acid in grooved slides and sealed in gold-size. Illustrations were drawn under microprojection in a dark chamber.

In following the courses of vascular bundles from the flower stalk to their entrance to different floral members we start with 12–14 vascular bundles in two rings, one set of bundles being opposite the ridges of the pedicel and the other set opposite the furrows. But before entering the receptacle (fused calyx and corolla) ten bundles, five in the outer and five in the inner circle, are noticed. At the base of the receptacle and below the three-lobed glandular pistillodium, these inner and outer vascular bundles develop branches and form more or less a ring of vascular tissue from the inner edges of which smaller and weaker branches develop and pass centrally to the pistillodium (Fig. 1). The vascular bundles of the pedicel meet at the base of the receptacle and form a sort of circular ring (siphonostele). From the outside of this ring fifteen traces in five groups (i.e. three traces in a group) pass to the sepals. A group of three traces passes to each sepal, the middle trace forming the sepal main and the two side ones constituting the sepal laterals. At a slightly higher level, or at almost the same level, and alternating with the groups of sepal traces, are those traces which become the main veins of the petals. Lateral petal traces are formed by branching of the petal mains higher up.

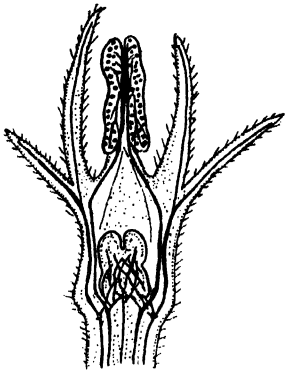

Fig. 1. Longisection of the male flower bud of *Lagenaria leucantha* showing courses of vascular bundles. × 4.

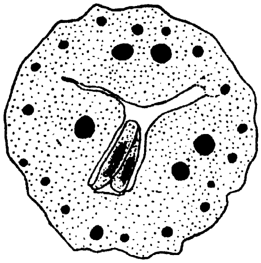

Fig. 2. Transverse section of the male flower bud of *Lagenaria leucantha* towards the base showing the filaments of the compound stamens containing two vascular bundles each and the filament of the simple stamen with one vascular bundle—filaments are still attached to the receptacle. × 60.

Towards the inner side of the vascular ring five bolder traces in 2 + 2 + 1 arrangement are seen passing through the inner side of the receptacle. These are the traces of the stamens. Two pairs of traces enter separately into two broader filaments and the odd trace passes to the odd narrower filament. The pair of traces of a broader (bithecal) stamen do not fuse but run parallel all along the filament and part of the connective and meet only at the upper portion of the connective. The filaments of the two larger stamens possess therefore two distinct vascular bundles from the base right up to the attachment of the anther to the connective (Fig. 2). Further on in the connective portion they branch off laterally to supply the sinuate theca and ultimately coalesce.

The inner five vascular bundles which actually share the three stamens in 2 + 2 + 1 arrangement thus represent the supply tissue for each theca (five thecae being formed out of three stamens [2] + [2] + [1]). The stamens are five in number in *Lagenaria* but four of them are fused in pairs forming two compound stamens, one remaining free. Formation of four stamens is not infrequently found in *Lagenaria* (Fig. 3). In the case where the stamens are four, each of three filaments carries a single vascular bundle and bears a single theca and one filament carries two vascular bundles. The theca is fairly long and involuted in more or less three folds over the broad and soft connective. Hence in a transverse section of a normal male flower of *L. leucantha* (6 + 6 + 3) or 15 cross-sections of the theca are found (Fig. 4). The thecae usually terminate with an inward bend; hence in section towards the base of the anther a thecal section is sometimes noticed in the central staminal column.

Each theca is divided centrally by a several-layered partition tissue which is narrower towards the apex and thus the theca is bisporangiate. The thecal partition wall breaks up at maturity at the narrower end giving rise to a confluent single microsporangium.

Naudin (1855) and Van Tieghem (1875) conceived three as the basic number of stamens in the Cucurbitaceae, while Eichler (1875) considers each stamen to have two (one larger and one smaller) vascular bundles and the larger bundles arranged face to face, the smaller bundles ultimately disappearing. Heimlich (1927), while studying the development and anatomy of the flowers of cucumber, also postulates the three-staminate nature of the male flower, but admitted that a comparative study of the stamens would throw light on its real morphology.

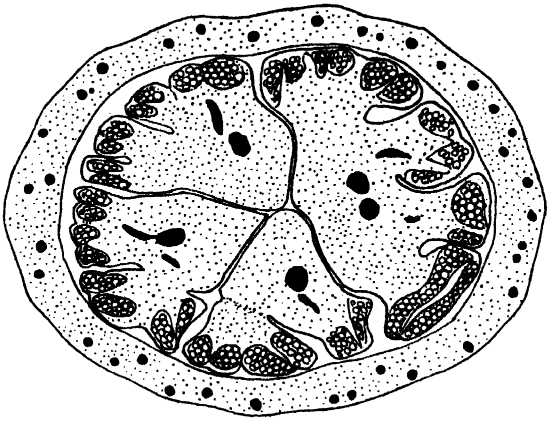

Fig. 3. Transverse section of the male flower bud of *Lagenaria leucantha* at the upper level showing four stamens, three with a single vascular bundle in each and the other with two vascular bundles. All vascular bundles show branching in the connective region. ×40.

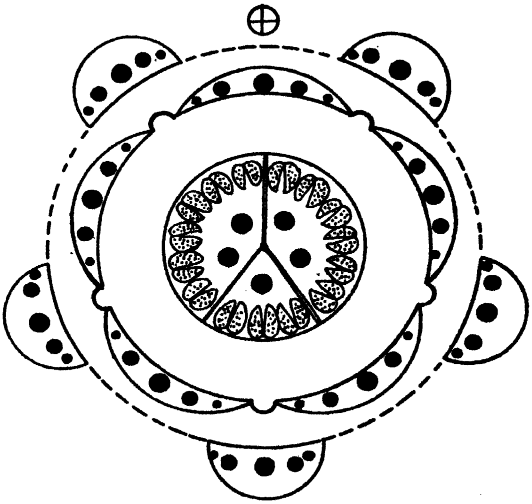

Fig. 4. Vascular floral diagram of the male flower in *Lagenaria leucantha*, two stamens sharing two vascular bundles and one stamen with one vascular bundle.

The existence of five bithecal stamens in certain species (*Telfaira*), the presence of five free monothecal stamens in a number of genera, and the occurrence of five- or three-staminate flowers in the same species (*Luffa cylindrica* (Linn.) Roem.), together with various modifications in stamens* in this interesting family, makes it difficult to accept three as the basic number and five as merely the number formed owing to splitting up (Naudin, 1855) of the so-called complete stamens. I have taken up the comparative study of about fourteen species covering about ten genera, and I think, as suggested by Willis (1919), Saunders (1937) and Wettstein (1924), that Cucurbitaceae is definitely pentamerous with a tendency to reduction to trimery. Pentamery is evident in the two accessory whorls, the calyx and the corolla. In the third whorl, the androecium, a gradual reduction from pentamery to trimery can be clearly traced. The tricarpellary pistil is however more or less permanently fixed, though two- (*Wilbrandia*) or four- (sometimes in *Cucumis*) or five- (*Cucurbitella*) placentiferous ovaries are found. The species of the genera like *Lagenaria*, *Cucurbita*, *Benincasa*, *Momordica*, *Luffa*, *Colocynthis* (*Citrullus*), *Cucumis*, *Sechium*, *Melothria*, *Coccinia*, etc. (many of them cultivated), are three-staminate, whereas the species of the genera *Fevillea*, *Anisosperma*, *Alsomitra*, *Hemsleya*, *Zanonia*, *Siolmatra*, *Gomphogyne*, *Thladiantha*, etc. (mostly wild), are five-staminate, the stamens being free and monothecal. The reduction or suppression of an organ (a stamen) or part of an organ (a theca) can both be imagined to take place either simultaneously or in succession in this family. Suppression of a theca of a normal five-staminate bithecal anther may perhaps occur earlier than the subsequent reduction in number of stamens. From a careful examination of a number of materials, I notice that stamens of five-staminate genera are complete in themselves (see Torrey & Gray, 1838–40), except for the suppression of a theca. By union of a pair of these monothecal stamens, three-staminate (two compound, one simple) forms have been derived. Hence it would be wrong to suggest that Cucurbitaceae is 2½-staminate. The question of splitting of a vascular bundle into two is not admissible, because in *Lagenaria leucantha* two distinct vascular bundles in the filaments apart from one another and surrounded by parenchymatous tissue are clear. In *Benincasa hispida* (Thunb.) Cogn. and occasionally in *Coccinia cordifolia* (Linn.) Cogn. three bithecal stamens are found; the third bithecal stamen should best be regarded here as a reversion into the normal ancestral habit. This third stamen, though morphologically similar to the other two, is smaller in size and contains only a single vascular bundle from the base up to the apex, whereas in the other two, two separate or fused vascular bundles are traceable. It would be worth attempting to cultivate five-staminate wild forms to see whether they ever give rise to three-staminate forms.

A complete comparative morphology of the male flowers of a number of species of Cucurbitaceae is in preparation. The results will be published elsewhere.

In conclusion, I offer my thanks to Dr H. Hamshaw Thomas, under whose supervision this work has been completed.

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* Unistaminate in *Cyclanthera*, bistaminate in *Luffa tuberosa* Roxb. and monadelphous in *Cucurbita*.

REFERENCES

(Including works containing a systematic account of the Cucurbitaceae but not cited by name in the text)

BRITTON, N. L. & BROWN, A. (1898). *Illustrated Flora of the Northern States and Canada*, **3**, 249. New York.
CLARKE, C. B. (1879). In Hooker's *Flora of British India*, **2**, 604. London.
EICHLER, A. W. (1875). *Blüthendiagramme construirt und erlaütert*, Theil Erster, pp. 302–20. Leipzig.
GAMBLE, J. S. (1935). *Flora of the Presidency of Madras*, **1**, 526. London.
GRAY, A. (1908). *New Manual of Botany*, 7th ed. (revised by B. L. Robinson and M. L. Fernald), p. 764. New York.
HEIMLICH, L. F. (1927). The development and anatomy of the staminate flowers in cucumber. *Amer. J. Bot.* **14**, 227–35.
LEE, B. (1937). *Microtomists Vade Mecum* (edited by GATENBY, J. B. & PAINTER, S.), p. 677, no. 135. London.
NAUDIN, C. (1855). Organographie Vegetale. Observations relatives a la nature des vrilles et à la structure de la fleur chez les Cucurbitacées. *Ann. Sci. Nat.* (Partie Bot.), IV, **4**, 11–17.
RIDLEY, H. N. (1922). *Flora of Malay Peninsula*, **1**, 842. London.
SAUNDERS, E. R. (1937). *Floral Morphology*, **2**, 489. Cambridge.
SMALL, J. K. (1913). *Flora of the Southern United States*, 2nd ed., p. 1134. New York.
TORREY, J. & GRAY, A. (1838–40). *A Flora of North America*, **1**, 539.
VAN TIEGHEM, P. (1875). Recherches sur la structure du pistel et sur l'anatomie comparée de la fleur. *Mem. Acad. Sci. Inst. Fr.* **21**, 1–262.
WARMING, E. (1890). *Handbuch der Systematischen Botanik*, p. 352. Berlin.
WETTSTEIN, R. (1924). *Handbuch der Systematischen Botanik*, p. 834. Leipzig.
WILLIS, J. C. (1919). *A Dictionary of the Flowering Plants and Ferns*, p. 192. Cambridge.

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NEW OR RARE BRITISH CHRYSOPHYCEAE. I.

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(With 3 figures in the text)

The English Lake District contains many more or less peaty small bodies of water which are rich in undescribed or rare Chrysophyceae. It is commonly considered that members of this class favour cold water. While they often occur in winter, they are by no means confined to the colder months of the year. For example, *Uroglena americana* Calkins, a very common plankton alga in the English Lake District, has occurred in abundance at 20° C. and is absent in the colder months, while some of the Chrysophyceae described in Lund (1942*a*, *b*) grew actively at over 20° C. and even as high as 31° C. Moreover, *Chrysocapsa epiphytica* n.sp., *Chrysoglena verrucosa* Wislouch and *Synura sphagnicola* Korsch. grow in the laboratory between 16 and 20° C. *Mallomonas akrokomos** Ruttner, though commonly considered as occurring in winter (Lund, 1937), may have its maximum in midsummer in the English Lake District and elsewhere (e.g. Thompson, 1946). In my opinion, it is much more probable that the chemical constitution of the water is the most important factor influencing their seasonal distribution.

In the following, where the alga concerned was only found in one or a few localities, these are followed by the full National Grid reference (Ordnance Survey, 1947), the system of which is explained on all the maps.

(1) *Chrysostenosphaera globulifera* Scherffel. Rare in Nor Moss Tarn, Claife Heights, Lancashire (34/366991).

(2) *Cyclonexis erinus* Jane (1939–40) occurred in abundance in the plankton of Wise E'en Tarn, Colthouse Heights, Lancashire (34/368977) in April 1948. Some of the cells were shorter (11 μ length) and others broader (9 μ breadth) than those described by Jane.

(3) *Synura sphagnicola* Korsch. Dr P. Bourrelly has kindly confirmed this alga. It is widespread in peat pools, particularly those with marginal or submerged *Sphagnum* and is equally abundant in the mountainous central area of the English Lake District and in the low-lying peat 'mosses' to the south.

The previous records of these three algae in Great Britain are given in Jane (1945).

(4) *Chrysoglena verrucosa* Wislouch (1914). Fig. 1A–N. In August 1946 I received from Mr E. D. Le Cren samples of water from two peat pools rich in submerged *Sphagnum* and lying on Birker Fell, Cumberland, in the neighbourhood of Devoke Water (34/1697). These contained a flagellate resembling *Microglena* Ehr. in the numerous superficial verrucae; they were, however, unsilicified and the organism appears to be identical with *Chrysoglena verrucosa* Wislouch (1914), only recorded from near Leningrad (Petrograd), Russia. In the last two years I have received material from Dr H. M. Canter and found it myself in several localities at diverse times of year. In the English Lake District, it occurs both in the mountains and in the lowland peat 'mosses' on the southern margin. In all cases it occurred in peat pools, often with submerged as well as marginal *Sphagnum* and often accompanied by *Synura sphagnicola* Korsch., *Gloeomonas* sp. and *Cryptomonas* sp.

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* Mis-spelt *akromonas* in Lund (1937) and *akrokomas* in Thompson (1946).

The English specimens (29–43 μ length; 13–27 μ breadth, one cell 22 μ length; 11 μ breadth) do not reach the maximum length of the Russian (30–52 μ), while only the subspherical ones are as broad (Russian; 20–25 μ). However, Wislouch's figures (1914, pl. 3, figs. 14, 21) suggest that some cells are smaller than the recorded dimensions, and

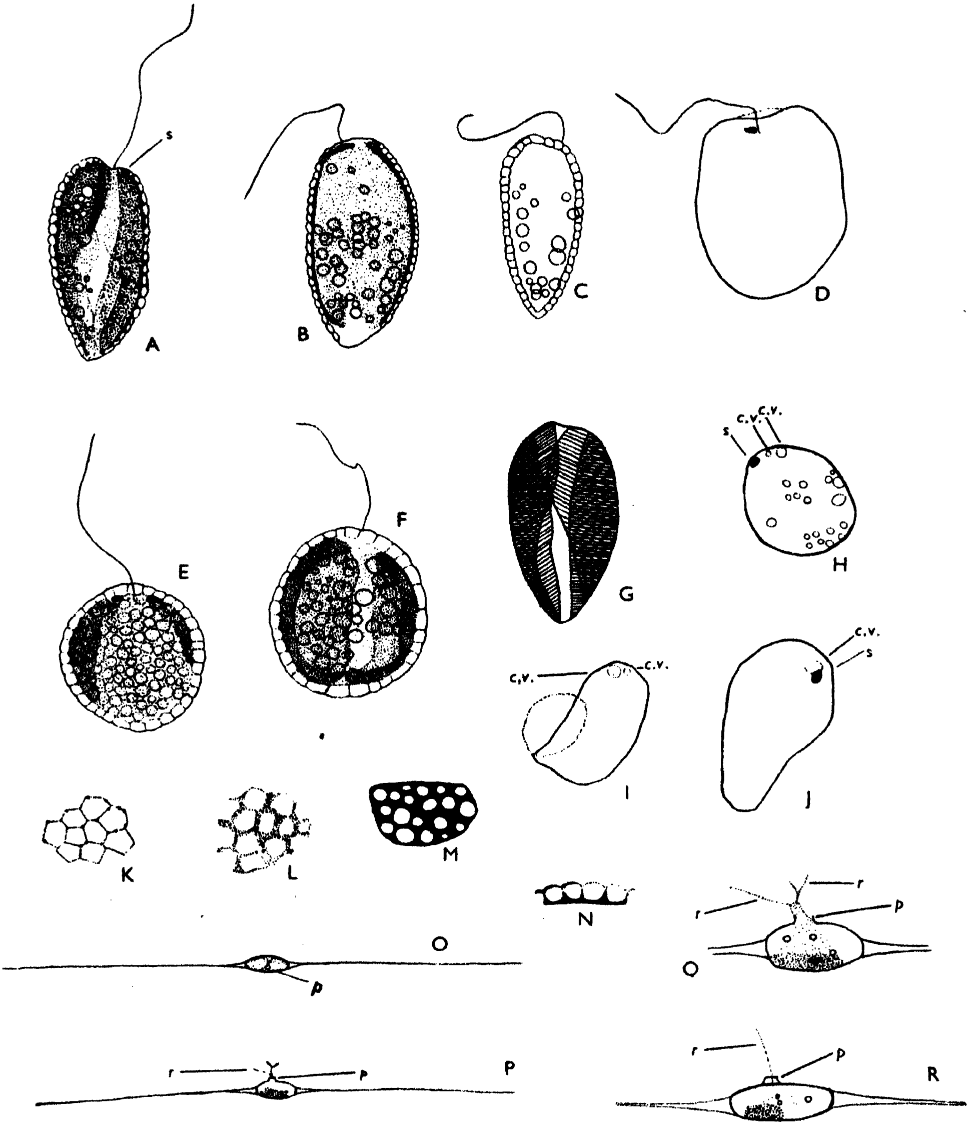

Fig. 1. A–N, *Chrysoglena verrucosa* Wislouch; A–F, free-swimming cells; G, chromatophores; H–J metabolic cells showing contractile vacuoles; K–N, verrucae, K–M from above, passing from outer surface of periplast (K) to outer surface of chromatophore (M), N, in optical transverse section. O–R, *Diceras longispinum* n.sp.; O, cell from above, P, from the side, Q, R, envelope with protoplast. All from material killed in 2 % osmic acid. *c.v.* contractile vacuole; *p*, pore; *r*, rhizopodium; *s*, stigma. A–J, × 770; K–N, Q, R, × 1125; O–P, × 425.

young daughter cells are about half the size of the parent. It is necessary to measure free-swimming individuals, since those in contact with the substratum may exhibit considerable metaboly (Fig. 1 H–J). In motion, the cells are usually elongate (Fig. 1 A–C), though some are almost spherical (Fig. 1 E, F). The single flagellum is inserted in a small anterior concavity of varying prominence, and is approximately the length of the cell. The verrucae (Fig. 1 K–N) form a beaded margin to the cell in optical section (cf. Wislouch, 1914, pl. 3, fig. 11), being convex on the outer and inner margins, while the divisions between them are at right angles to the surface of the cell. The inner margin is partly enmeshed in the chromatophore which, consequently, has a serrate margin (Fig. 1 A, N). In surface view, at this level, the verrucae appear rounded (Fig. 1 M), but their outline becomes more angular towards the cell surface (Fig. 1 L, K). Similar verrucae are known in *Chromulina* (Pascher, 1913; Skuja, 1948) but, as in *Chrysoglena*, their nature and origin are not wholly clear. In the present specimens, they form a firm vacuolated protoplast network without any calcification or silicification. They disappear on heating and on the death and disintegration of the cells in water. In the latter case, the partitions between the vacuoles break down to form larger, more irregular ones. Finally, the surface of the cell becomes smooth so that it is indistinguishable from a *Chromulina* (cf. also *C. erkensis* Skuja, 1948, p. 243). Healthy cells without verrucae have never been seen.

There are two simple anterior contractile vacuoles (Fig. 1 H, I; *c.v.*), to the side of which lies the stigma (Wislouch, 1914, records one or two contractile vacuoles and stigmata). Leucosin and oil are stored. Wislouch records one chromatophore but his figures (1914, pl. 3, figs. 10, 12–14) show that there is a slit down one side at least. In the English specimens there appear to be two chromatophores (Fig. 1 A, G), though they sometimes overlap one another to a degree which makes it uncertain as to whether they are or are not connected at one or other end of the cell. Each chromatophore covers the face of the cell in a plane (lateral) at right angles to that in which the flagellum is inserted. Thus the cell viewed from either side appears to have one unbroken chromatophore (Fig. 1 B). In the plane of the flagellum (dorsoventral) there is an irregular gap between the chromatophores (Fig. 1 A, G).

Though the alga has been kept alive in the laboratory for a year in a mixed algal culture no cysts have been observed in it or in nature. For the Russian material, Wislouch describes cyst formation. The typical chrysophycean cysts formed have a smooth wall and one pore closed by a conical plug. *Chrysoglena* differs from *Microglena* Ehr. (Conrad, 1927) in the type of verrucae and the simple anterior contractile vacuoles. Several species of *Chromulina* possess similar, though scattered, verrucae (e.g. *C. stellata* Pascher, *C. Pascheri* Hofender (Pascher, 1913), *C. erkensis* Skuja (Skuja, 1948)), and *Chrysoglena* only differs from these in that the whole periplast is regularly covered with verrucae, and the cells are larger and more robust. Wislouch (1914), Conrad (1927) and Hüber-Pestalozzi (1941) include *Chrysoglena* in the Mallomonadaceae of Pascher (1913). In view of the simple vacuolar apparatus, the absence of silicification and similarity of verrucae to those of *Chromulina* spp., it would appear more correct to place *Chrysoglena* in Fritsch's (1935, p. 555) Chromulinaceae (Euchromulinaceae of Pascher, 1913, p. 11).

(5) *Diceras longispinum* n.sp. (Fig. 1 O–R). The protoplast is lodged in an elongate oval envelope (10–12μ length; 4·5–5·0μ breadth) with a small lateral pore which is produced above the surface of the envelope as a cylindrical collar. Attached to each end of the envelope, in the plane of the long axis, is a long spine. The spines are of equal length

(53–65 μ), relatively wide where they join the envelope and extremely fine for the greater part of their length (Fig. 1 O, P). Owing to their delicacy, they can become somewhat bent if damaged. Neither the envelope nor spines give positive reactions for cellulose. The protoplast usually fills the envelope, and a simple or branched rhizopodium passes through the pore (Fig. 1 Q, R). The latter is reminiscent of the two flagella recorded for *D. Chodatii* Reverdin (1919, fig. 29). In some cells, no rhizopodium could be detected and, in others, a small portion of the protoplast bearing rhizopodial threads lies outside the pore (Fig. 1 Q). No flagellum, contractile vacuoles or stigma are present. There is a single parietal chromatophore. Oil and leucosin are stored.

Occasionally division of the protoplast was seen, but the fate of the two daughter cells is unknown. As in other members of the genus, no cysts were seen. Type material is lodged with the Freshwater Biological Association.

D. longispinum occurred in moderate numbers in the plankton of Wise E'en Tarn, a small lake on Colthouse Heights, Lancashire (34/368977), in April 1948, accompanied by *Dinobryon divergens* Imhof, *Cyclonexis erinus* Jane and *Uroglena americana* Calkins. It is a very delicate alga, so that prolonged observation of living cells has not been possible under the microscope nor have attempts to culture it been successful.

The genus is new to Great Britain. A few specimens of another species have been observed in Blelham Tarn, Lancashire (34/3650), in 1947.

Four other species of *Diceras*, all planktonic, are known (Fott, 1936, p. 6), from all of which *D. longispinium* differs in the very long spines. *D. Chodatii* Reverdin (1919), the type species (envelope 14 μ length; 6 μ breadth; spines to 40 μ length), has ovoid cells with spines of unequal length, one standing at an angle to the other. The flagella recorded for this alga have not been observed in the other species. *D. ohridana* Fott (1933) has short spines (15–18 μ) of equal length, both bent in the same direction and making an angle of 125–135° with the long axis of the protoplast (4–10 μ length). The spines narrow relatively gradually from a wide base. No pore to the envelope is recorded. *D. phaseolus* Fott (1936) has wide ellipsoid to bean-shaped envelopes (5–7 μ length). The pore lies in the concavity of the bean-shaped envelope. The spines are of equal length (25 μ), taper gradually and lie in the plane of the longitudinal axis of the envelope or may be a little bent. There are two contractile vacuoles. *D. ollula* Fott (1936) differs from the other species in that the cells sometimes have three spines (25–32 μ length) lying in different planes. Where two spines are present, they are commonly directed backwards from the pore. The envelope (6·5–8·5 μ diam.) is widely oval to subspherical. There are two contractile vacuoles. In the last two species no rhizopodia are recorded. In *D. Chodatii* Reverdin (1919) the envelope alone is composed of cellulose as he states is also the case in *Dinobryon*.

Chrysocapsa epiphytica n.sp. (Figs. 2 and 3). The cells (7·5–15 μ length; 7·5–12 μ breadth) are embedded in mucilage to form pale brown epiphytic colonies of varying size and shape (Figs. 2 A, 3 E). Young microscopic colonies are spherical, and even colonies of 2·5 cm. diameter may be approximately so. The larger colonies (Fig. 3 E) are, however, usually more or less lobed or even weakly foliose, reaching 5·5 cm. long. The surface lobing is often reminiscent of that of the human brain. The cells are nearly all aggregated in the surface of the mucilage (Fig. 2 C) in an irregular layer about two to four cells deep. They are widely oval to subspherical with their long axes at right angles to the circumference of the colony. Young (Fig. 3 F–K) are more oval than old cells, enlargement after division being mainly or wholly in breadth. While the majority of the cells are 8–12 μ long and slightly less broad, a few reach 15μ length by 12μ breadth. These latter are usually oval or even weakly ovoid (Fig. 3 D), and lie within the surface zone in which most of the cells are aggregated. They do not appear to serve any special function.

The cell wall is not clearly distinguishable in ordinary living cells. As division proceeds it gelatinizes so that the whole cell appears to divide. In akinetes it is prominent (see below). It is clearly visible in empty cells (Fig. 3 M). In plasmolysing solutions and several stains, irregular lines become visible about half-way between adjacent cells and may form a faint polyonal network (Fig. 2 B). In some living colonies kept in ferruginous water these lines also appeared and are also occasionally visible in colonies mounted in indian ink (Fig. 3 L). They appear to be the remnants of the mother-cell walls which have not become so diffluent as the main mass of mucilage, since, in dividing or newly separated cells, they form a spherical investment (Fig. 3 M, N). In alcohol and other substances, which cause massive contraction of the mucilage, the cells may become tightly packed and polyonal due to mutual pressure.

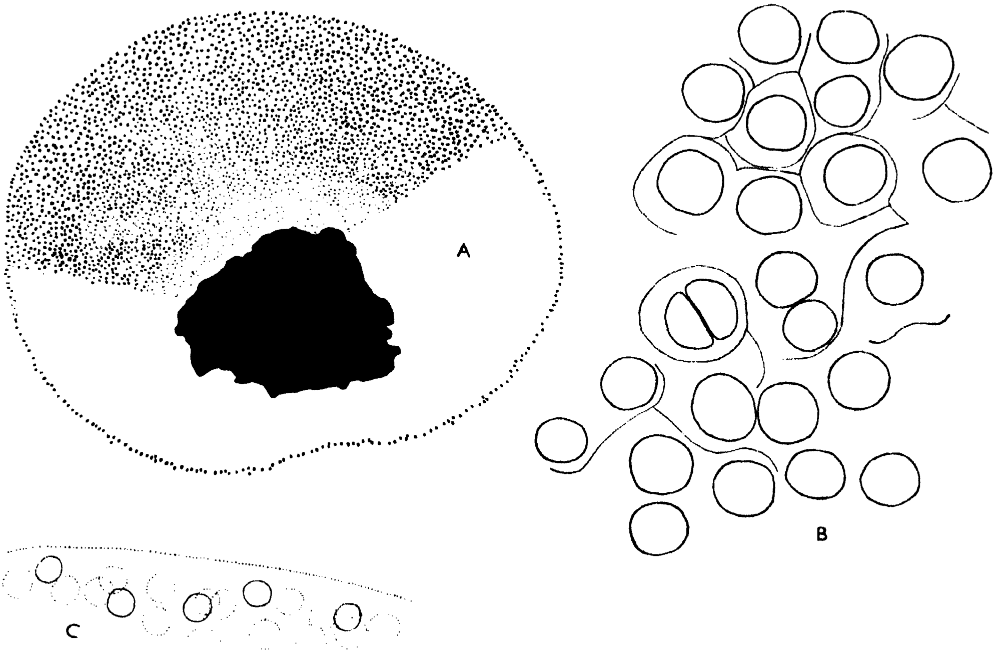

Fig. 2. *Chrysocapsa epiphytica* n.sp. A, colony (from photograph); B, portion of colony in 10% $CaCl_2$; C, margin of colony in optical transverse section. A × 40; B × 775; C × 380.

There are usually two (especially in recently divided cells) or four parietal chromatophores (Fig. 3 A). Commonly, the four chromatophores lie so close together that it is not clear whether or not they fuse in the equatorial region, though they are clearly separated at the poles of the cell. One or more vacuoles containing moving granules are sometimes present. There are no contractile vacuoles or stigma. Fatty materials are the commonest storage products; leucosin occurs and also an angular reserve of unknown nature (Fig. 3 C).

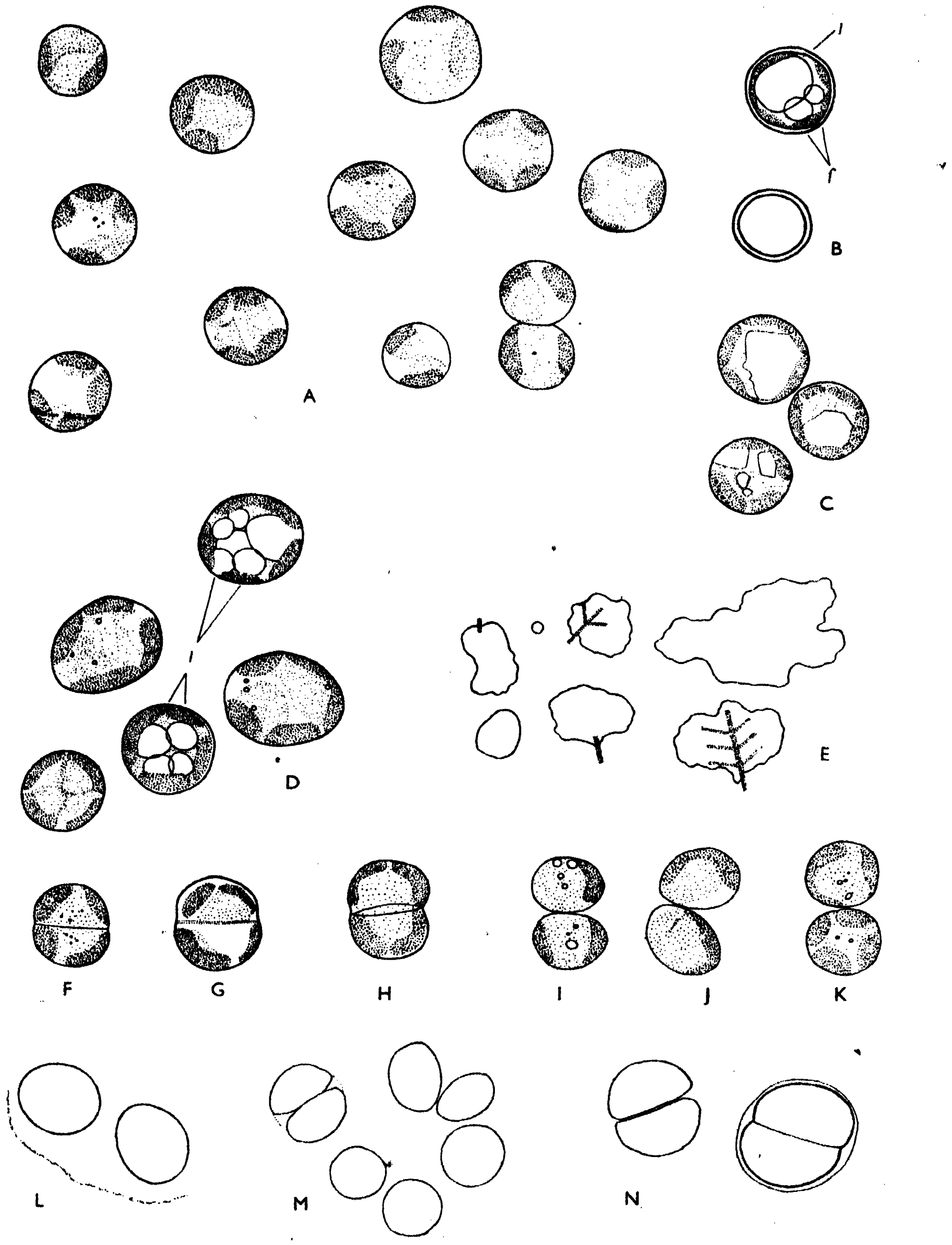

Fig. 3. *Chrysocapsa epiphytica* n.sp. A, cells from outer portion of colony; B, living and dead akinetes; C, cells with angular reserve product; D, large cells from inner part of colony; E, variously shaped colonies, some still attached; F–L, dividing and recently divided cells, L in indian ink; M, empty cells; N, dividing cells in 10% $CaCl_2$. A–L, from live material, *f*, fatty products, *l*, leucosin; E half natural size; the rest, × 1125.

The only form of reproduction seen (Fig. 3 F–K) is the formation of two non-motile daughter cells (? aplanospores). No cysts have been seen. Their place is perhaps taken by the thick-walled resting cells (akinetes). These are the same size as the vegetative cells and have only been observed in material kept for a prolonged period in the laboratory without enrichment (Fig. 3 B). If the colonies are subcultured at intervals, vegetative growth continues. The akinetes appear prominent because of the refractive nature of the walls and their richness in reserve products. The walls do not contain silica and give well-marked cellulose reactions, whereas those of the other cells rarely give a positive reaction and, in the colonies where this was so, the presence of akinetes made it unclear whether these cells were not themselves in the process of becoming akinetes. The mucilage of the colony stains faintly violet in chlor-zinc-iodide. No zoospores occurred. It seems possible that they are absent, though the products of the germination of the akinetes are unknown.

Plentiful material of *C. epiphytica* has been examined, and colonies kept under diverse conditions in the hope of inducing zoospore formation. Unlike most Chrysophyceae, this alga can be kept alive for months in the laboratory, and for a week or more on a slide under a cover-glass.

C. epiphytica was discovered by Dr J. L. Harley in Blelham Beck in the vicinity of Congo Bay, Windermere (34/369021), in March 1948. It increased in numbers and average colony size until the middle of May 1948, but had completely disappeared by the end of the month, perhaps owing to the dry weather. While common on the divided leaves of *Ranunculus* and *Apium* it never occurred on the abundant shoots of *Phragmites*, *Equisetum*, *Carex* and *Scirpus*. One dead twig had several colonies on it. Another large palmelloid Chrysophyceae often accompanied it.

This differs from all other species in the colony size, walled cells and akinetes. *Chrysocapsa maxima* Geitler (1926) is epiphytic with colonies up to 5 mm. diam., has a similar arrangement of the cells in the colony and multiplication by division form two daughter cells. Cysts and zoospores are unknown though, according to Geitler, the latter probably do occur. The cells (10–16·5 μ diam.) are naked, possess one parietal chromatophore and contractile vacuoles. *Celloniella* (Pascher, 1929) produces similar colonies under certain conditions but differs markedly in other characteristics. Most Chrysomonadales with the non-motile stage in the life history dominant (Chrysocapsineae, Fritsch, 1935; Chrysocapsales, Pascher, 1925) have naked cells, often containing contractile vacuoles and sometimes stigmata. Any cell may become a swarmer by development of a flagellum so that it is often doubtful whether a species should be placed in this suborder or Chrysomonadineae (suborder of Chrysomonadales, Fritsch, 1935; Chrysomonadales of Pascher, 1925). In type of cell and reproduction *C. epiphytica* resembles members of the Chysosphaerales (Pascher, 1925; Fritsch, 1935) and, in colony, the Chrysomonadales (*sensu* Fritsch, 1935). The exact limits of *Chrysocapsa* are still uncertain. Pascher (1925) would restrict the genus to species with chromulinoid swarmers though he points out (Pascher, 1925, p. 549) that zoospores are rarely seen in the genus (the same applies to cysts). Should *C. epiphytica* be found to have ochromonad swarmers, it should possibly be placed in *Phaeogloea* Chod. The cells of this genus, placed in the Chrysocapsineae by Fritsch (1935) and Chrysosphaerales by Pascher (1925, p. 562), are walled. If the akinetes take the place of the typical Chrysophycean cyst and motile stages are absent in *Chrysocapsa epiphytica*, it can hardly be retained in either genus.

I am retaining the present alga in *Chrysocapsa* on account of the type of colony and ill-defined cell wall (except in the akinetes).

Type material and photographs taken by Dr H. M. Canter of *C. epiphytica* are lodged with the Freshwater Biological Association.

REFERENCES

CONRAD, W. (1927). Le genre *Microglena* C. G. Ehrenberg (1838). *Arch. Protistenk.* **60**, 415–39.

FOTT, B. (1933). Die Schwebeflora des Ohrid-Sees. *Bull. Inst. bot. Univ. Beograd.* **2**, 153–75.

FOTT, B. (1936). Dva nové druhy rodu *Diceras* Reverdin. *Věst. Král. Česke Společn. Nauk*, **2**, 1–7. (Czech with German résumé.)

FRITSCH, F. E. (1935). *The Structure and Reproduction of the Algae*, **1**. Cambridge.

GEITLER, L. (1926). Zwei neue Chrysophyceen und eine neue 'Syncyanose' aus den Lunzer Untersee. *Arch. Protistenk.* **56**, 291–4.

HÜBER-PESTALOZZI, G. (1941). Das Phytoplankton des Süsswassers. Systematik und Biologie. 2 Teil, 1 Hälfte. Chrysophyceae, farbloser Flagellaten, Heterokonten. *Binnengewässer*, **16**. Stuttgart.

JANE, F. W. (1939–40). Two new Chrysophycean flagellates—*Cyclonexis erinus* and *Synochromonas elaeochrus*. *Proc. Linn. Soc. Lond.* 152 Sess. (1939–40), pp. 298–309.

JANE, F. W. (1945). Some new or little known algae from Anglesey and Caernarvonshire. *Trans. Anglesea Antiq. Soc. Fld Cl.*, pp. 73–90.

LUND, J. W. G. (1937). Contributions to our knowledge of British algae. VI: Some new British algal records I. *J. Bot., Lond.*, **75**, 305–14.

LUND, J. W. G. (1942*a*). The marginal algae of certain ponds with special reference to the bottom deposits. *J. Ecol.* **30**, 245–83.

LUND, J. W. G. (1942*b*). Contributions to our knowledge of British Chrysophyceae. *New Phytol.* **41**, 274–92.

ORDNANCE SURVEY OF ENGLAND AND WALES. Rev. 1920 (1947). Chessington.

PASCHER, A. (1913). Flagellatae. II. *Süsswasser-flora Deutschlands, Österreichs und der Schweiz*, **2**. Jena.

PASCHER, A. (1925). Die braune Algenreihe der Chrysophyceen. *Arch. Protistenk.* **52**, 489–564.

PASCHER, A. (1929). Über die Beziehungen zwischen Lagerform und Standortsverhältnissen bei einer Gallertalge (Chrysocapsale). *Arch. Protistenk.* **68**, 637–68.

REVERDIN, L. (1919). Étude phytoplanctonique, expérimentale et descriptive des eaux du lac de Genève. *Arch. Sci. Phys. Nat.* **1**, 1–96.

SKUJA, H. (1948). Taxonomie des Phytoplanktons einiger Seen in Uppland, Schweden. *Symb. bot. upsaliens.* **9**, 3–399.

THOMPSON, R. W. S. (1946). The biology of Ladybower Reservoir (during the period of its first filling, April 1943 to December 1944). *Trans. Instn Wat. Engrs, Lond.*, **51**, 69–96.

WISLOUCH, S. M. (1914). Sur les Chrysomonadines des environs de Pétrograd. *J. Microbiol.* **1**, 251–78. Pétrograd (Russian with French résumé).

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REVIEWS

British Mycological Society, 1896-1946: *Proceedings of the Jubilee Meeting held in London, 20-25 October*, 1946. Comprising vol. xxx of the Society's *Transactions*, 1948. 9¾ by 6¼ in. Pp. 208, with 8 plates and 2 portraits. Cambridge University Press. Price 40*s*.

Mycologists everywhere, and a great many others besides, will welcome the appearance of this volume which marks the culmination of the British Mycological Society's Jubilee anniversary celebrations. Apart from their social and domestic functions, the proceedings were designed in the main to show the diverse relationships of mycology and to stress its international significance; and the success with which these objects were attained is now made evident. Thus, 139 pages in the volume under review are devoted to papers which are grouped under the five headings of medical mycology (C. W. Emmons) and mould products (J. H. Birkinshaw); plant pathology as represented by the study of seed-borne fungi (Miss L. Doyer, A. E. Muskett and Miss Mary Noble); mycorrhiza (J. E. B. Melin) and soil fungi (C. G. C. Chesters); growth-factor requirements (Nils Fries and Miss L. E. Hawker); and taxonomy (Miss M. A. Brett, Miss E. M. Wakefield and Roger Heim). The list of contributors is a notable one, the more so for the inclusion of a number of eminent foreign mycologists whose work has not previously been readily available to students in this country. To this series of papers must be added the Presidential Address (J. Ramsbottom), affording a survey of past and present mycological problems as seen by the taxonomist whose ideal is 'an epitome of our knowledge' of fungi; and, by the same author, the interesting introductory account of the Society and its earlier activities. Dr Ramsbottom also, as is fitting, supplies the memorial of the late Carleton Rea, with which the main contents of the volume end.

Mycology is portrayed in these pages as of rapidly increasing significance, for biology at large and for human welfare. This Jubilee volume will doubtless attract many new workers to the subject: it deserves the widest possible circulation.

T. E. T. BOND

Vernalization and Photoperiodism. A Symposium. By A. E. MURNEEK, R. O. WHYTE *et al.* (*Lotsya*, vol. 1). Pp. 196. Waltham, Mass.: The Chronica Botanica Co.; London, W.C. 2: Wm. Dawson and Sons Ltd. $4.50.

The renewed interest in morphogenesis marks a reaction from the severely analytical approach to the problems of plant physiology, in which the primary physiological functions are considered in isolation. There are signs of a return to a more specifically biological outlook which aims at a study of the integration of function issuing in the behaviour of the plant as a whole and its adjustment to the environment. The impetus in this direction dates from the work of Gassner in 1918 on the after-effects of temperature during germination on the flowering behaviour of the winter varieties of cereals, and the discovery by Garner and Allard in 1920 of control of flowering by length of day. These challenging discoveries laid the foundations of studies in vernalization and photoperiodism. A vast literature has in the meantime appeared covering these fields of study, and the aim of the present symposium is to present an authoritative account of the present state of knowledge. Contributions have been made by some outstanding workers, and though, owing to delay in publication due to the war, the original aim has not been altogether achieved, nevertheless, the book is a valuable introduction to the subject and is fully documented.

The symposium opens with historical surveys of vernalization and photoperiodism by Whyte and Murneek which present the salient features of the problems. Vernalization research is dealt with regionally, and a full account is given of the work of the Soviet agronomists, including the technique introduced by Lysenko for application in agriculture. The present views on the practical value of this technique are fairly stated. The analytical study of the vernalization process carried out in this country is presented in detail, but, perhaps wisely, the theoretical aspects are not unduly stressed. The only serious omission is the work of Melchers and Lang on the vernalization of biennials, but reference is made to this later in the paper by Hamner. The review of photoperiodism includes a summary account of theories on the mechanism of the length of day effect.

The evidence for the hormonal nature of the underlying mechanisms, derived from grafting and transmission experiments, is reviewed in a paper by Hamner, who contributed so much to the analytical study of photoperiodism. Borthwick, Parker and Hendricks give an account of their work on the photochemistry of the photoperiodic reaction and stress the similarity of the 'action spectrum' of flower initiation to that of leaf and stem growth reactions in etiolated seedlings. An interesting suggestion of a single-hormone mechanism similar in long and short day plants is put forward, the specific reactions of the two types of plants depending upon sub- and supraoptimal concentrations of a substance formed in the leaves and destroyed by light. Among the other contributions a fascinating paper by Roberts and Struckmeyer may be cited which presents irrefutable evidence, based on the examination of seventy species, that the first reaction leading to flower initiation takes place in the cambium, the activity of which is curtailed resulting in 'maturation changes' in phloem and xylem. Such a reaction in the cambium is seen in all cases of response irrespective of whether long and short day plants are in question or whether flower initiation results from change in daylength, 'temperature girdles' or mechanical compression of the phloem. Murneek in a further paper discusses the nutritional relations of photoperiodism and disposes, it would appear, of the carbohydrate/nitrogen ratio as a factor in flower initiation. Allard has contributed a speculative paper on the role of length of day in past geological epochs and its relation to distribution of plants in fossil floras. Finally, a valuable contribution by Went discusses the effects of diurnal changes in temperature during light and darkness to which the name thermoperiodicity is given.

Three supplements in German are appended, two by Bünning, of which one deals with photoperiodism in the tropics and the other presents a welcome summary of his theory of photoperiodic response dependent upon the endogenous diurnal rhythms in plants. The genetics of photoperiodism and vernalization are dealt with in a supplement by Lang. The symposium is introduced by a foreword from Thimann who, as an authority on plant hormones, discusses the present position and future prospects of research from this angle.

Attention should be drawn to a serious error on p. 24 in discussing the auxin relations of the rye grain; the statement should read '*No* auxin is detectable in the embryo...etc.' which reverses the sense of the statement and resolves a flagrant contradiction in the text.

F. G. GREGORY

Flora Malesiana. Series 1, vol. 4, *Spermatophyta*, part 1. 9¾ × 6½ in. Pp. i–xl (Introduction, General Considerations), 1–39 (Taxonomic Revisions), 1–24 (Sample treatment of vol. 1, special part), 1–10 (Sample treatment of vol. 2), 1–6 (Sample treatment of vol. 3); 1 plate and 71 text-figures. Batavia: Noordhoff-Kolff N.V. 1948. 1 guilder Netherlands pro 16 printed pages (averaging 240 pages per annum). (Waltham, Mass., U.S.A.: Chronica Botanica Co.; Groningen, Holland: N.V. Erven P. Noordhoff. General Editor: Dr C. G. G. J. Van Steenis, Rijksherbarium, Leiden, Holland.)

The publication of the first part of this work, under the auspices of the governments of the Netherlands and of Indonesia, three years after the liberation of south-east Asia, is a sign of the vigour with which our Dutch colleagues have returned to the task of promoting botanical science in Malaysia. The work was projected by the General Editor, Dr Van Steenis, long ago, studied and fostered by him during nearly four years of internment, and now, through his determination and perseverance, is taking shape as the modern foundation of botany in south-east Asia.

Malaysia is understood to cover the great stretch of islands from (and including) the Malay Peninsula to the Torres Straits, and from the northernmost Philippines to Christmas Island in the Indian Ocean. It embraces the evergreen forest which lies between the monsoon forests of continental Asia and Australasia south and east of New Guinea and the Bismarck Archipelago. There is no general flora of this region which, as the land of the Dipterocarps, contains the grandest forest in the world. And how much it is needed is well known to all who have made their contributions, great and small, local and extensive, in its preparation. The *Flora Malesiana* intends now to revise thoroughly these preliminary works, to knit them together in synonymy, and to add all that can be derived, systematically and ecologically, from recent collections and expeditions. We are promised within the compass of a quarter of a century some thirty volumes in five series, not only authoritative monographs from algae to spermatophytes, of which alone there are estimated to be 2400 genera and 30,000 species, but general descriptions of Malaysian plant life and geography, including tables of the distribution of 2200 indigenous genera (in MS.), and a catalogue of Malaysian botanical collectors and collections (ready for press). There is coming, sooner let us hope than Dr Van Steenis expects, such a mine of information for general, local, applied and herbarium-botany as no progressive botanical institute can afford to be without. We cannot, for instance, classify as yet the sago-palms, the bananas, the gingers, or the majority of the trees of this enormous flora except in a very tentative way replete with the errors of ill-judged endemism, and this magnificent store of plant science has still to be incorporated in general botanical thought.

This first part of the flora consists of a general introduction, written by Dr Van Steenis, from practical and philosophical points of view, a taxonomic part contributed by monographers from Buitenzorg, Leiden, Utrecht, Groningen, Göteborg, and Kew, and samples of three other volumes to show the high standard of accuracy and comprehensiveness which is to be maintained. There is a fine preface by Dr L. G. M. Baas-Becking (Director of the Buitenzorg Botanical Garden) which establishes the international basis on which the flora is to be prepared. It is contended that regional or family monographs must precede local floras and that the order of publication must be opportunist as critical monographers become available. Thus, neither alphabetical nor systematic order can be followed for the taxonomic work, as shown by the first series of small monographs on Aceraceae, Philydraceae, Ancistrocladaceae, Aponogetonaceae, Burmanniaceae, Sphenocleaceae, Nyssaceae, Sarcospermaceae, Stackhousiaceae, and Actinidiaceae. Small families will naturally come first, but let us hope that the work on subdivisions or even genera of the bigger families, as Leguminosae and Euphorbiaceae, is also being put in hand. We feel that the immediate result of this start will be the co-operation of botanists all over the world to assist the General Editor in the record production of the great undertaking he is so eminently fitted to direct.

E. J. H. CORNER

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